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Design of standardized molecular tools to analyze regulatory properties and biotechnological applications of the soil bacterium *Pseudomonas putida*

TESIS DOCTORAL

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To my parents Anacleto and Roberta, so far but incredibly close

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Fue tan largo el vuelo que al final, casi lo confundo con mi hogar…. (VM)

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ABBREVIATIONS

Ap	Ampicillin
AU	Arbitrary units
BA	Benzaldheyde
BCD2	Bicistronic design 2
Ben	Benzoate
Bp	Base pair
BP	Band pass
$CaCl_2$	Calcium chloride
CHPA	3-chloro-4-hydroxyphenylacetic acid
Cm	Chloramphenicol
Crc	Catabolite repression catabolism
DB	Database
DNA	Deoxyribonucleic acid
FC	Flow Cytometry
FISH	Fluorescent *in situ* hybridization
GFP	Green fluorescent protein
Glu	Glucose
Gm	Gentamycin
H	Hour
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	1000 base pairs
Km	Kanamycin
LB	Luria–Bertani
MCS	Multi-cloning site
m-xyl	*m*-xylene
M	Molar
Mg	Milligram
$MgSO_4$	Magnesium sulfate

Min	Minute
Ml	Milliliter
mM	Millimolar
msfGFP	Misfolding-superfolder green fluorescent protein
mRNA	Messenger RNA
Mut	Mutant
NAD(H)	Nicotinamide adenine dinucleotide
Nox	NADH oxidase
Nm	Nanometer
nM	Nanomolar
Nt	Nucleotide
o-xyl	*o*-xylene
OD	Optical density
PBS	Phosphate buffered saline
PC	Phase contrast
PCR	Polymerase chain reaction
RBS	Ribosome binding site
Rpm	Revolution per minute
RNA	Ribonucleic acid
RNAP	RNA polymerase
SDS	Sodium dodecyl sulfate
Sec	Second
Sm	Streptomycin
sRNA	Small RNA
Suc	Succinate
T1, T0	Transcriptional terminators
Tc	Tetracycline
TF	Transcriptional factor
TCA	Tricarboxylic acid cycle
Tol	Toluene

UAS	Upstream activating sequences
UTR	Untranslated region
UV	Ultraviolet light
wt	Wild-type
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
σ	Sigma factor of RNA polymerase
2,6 DCT	2,6-Dichlorotolluene
1,3,5 tMBe	1,3,5-trimetylbenzene
3MBA	3-methylbenzylalcohol
3MBz	3-methylbenzoate

ABSTRACT

The field of synthetic biology uses engineering principles to change or design *de novo* biological systems. Similar to engineering, synthetic biology employs standards to design and assemble genetic parts, devices and systems. The use of uniform molecular tools, which enables comparison of results obtained in different laboratories, requires adoption of common nomenclature and implements to develop platforms for data collection. Bacteria are an optimal system for reprogramming or re-implanting biological devices and *Escherichia coli* is a common reference strain for these tasks. Soil bacteria such as *Pseudomonas putida* are a valid alternative as a chassis because of their extreme adaptability to the external environment. *P. putida* KT2440, a strain derived from the wild type, toluene-degrading *P. putida* mt-2, is the most promising candidate for synthetic biology applications. Diverse genetic tools have been optimized for editing the *P. putida* KT2440 genome and include a collection of standardized vectors. This thesis describes a number of molecular tools designed for work with *P. putida* KT2440 to clarify some questions regarding its regulation and use for biotechnological applications. We first designed and validated standardized plasmid- and transposon-based tools that allowed detailed analysis of the transcriptional state of given promoters in single cells and in clonal populations. Second, we explored the dynamics of the XylR-*Pu* regulatory network, which in *P. putida* is specialized in degrading aromatic compounds. We specifically studied single-cell responses of the promoter to increased production of its regulator and to different inducers. In addition, we analyzed XylR production *in vivo* after Crc protein-mediated repression. Finally, we showed the potential of heterologous expression systems for designing catalytic biofilms with *P. putida* KT2440.

RESUMEN

La Biología Sintética se inspira en los principios de la ingeniería eléctrica e industrial para modificar o diseñar nuevos sistemas biológicos. Al igual que la ingeniería, la Biología Sintética emplea estándares para diseñar y obtener partes genéticas y con ellas generar módulos y sistemas. El uso de herramientas moleculares uniformadas permite comparar resultados en distintos laboratorios, pero requiere la adopción de una nomenclatura común y el desarrollo de plataformas genéticas compartidas. Las bacterias constituyen un sistema óptimo para re-programar o re-implantar módulos genéticos, y *Escherichia coli* es la cepa más utilizada para estas tareas. Las bacterias del suelo, como *Pseudomonas putida* son una alternativa en cuanto que presentan una gran adaptabilidad hacia el ambiente externo. *P. putida* KT2440 es una cepa derivada de un aislado silvestre que degrada tolueno (*P. putida* mt-2) y es el candidato a hospedador con más potencial para aplicaciones industriales y medioambientales de circuitos genéticos hechos con Biología Sintética. La presente Tesis describe un conjunto de herramientas moleculares diseñadas para trabajar con *P. putida* KT2440, con la finalidad de clarificar algunos aspectos sobre las regulación de algunas de sus propiedades y avanzar en su utilización en aplicaciones biotecnológicas. En primer lugar, esta Tesis propone el diseño y la validación de herramientas basadas en plásmidos y transposones estandarizados que permitan efectuar un análisis detallado sobre el estado de activación de promotores específicos en células únicas y en poblaciones. A continuación, se investigan la dinámica de la regulación del nodo transcripcional formado por el complejo XylR/*Pu,* que está implicado en la degradación de compuestos aromáticos en *P. putida*. En particular, se analizan las respuestas del promotor *Pu* en distintas condiciones fisiológicas, como el aumento de la producción de su regulador XylR y la presencia de diferentes inductores químicos. Se analiza además la producción de XylR *in vivo* cuando las células de *P. putida* KT2440 crecen en un régimen de represión catabólica mediado por la proteína Crc. Finalmente, se muestra el potencial del sistema de expresión ChnR/*PchnB* diseñado para obtener condicionalmente biopelículas de *P. putida* KT2440 capaces de degradar compuestos halogenados.

I. INTRODUCTION

Part of this chapter has been published as:

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1.1 The world of synthetic biology: de-constructing living organisms to combine new engineered systems

In the past decade, scientists have made real progress in the use of engineering principles that enable change or *de novo* design of biological systems, to understand how they work for developing needs as drugs, chemicals, materials, energy sources and new instruments for data storage and information processing (Andrianantoandro et al., 2006; Brophy and Voigt, 2014; Cheng and Lu, 2012; Heinemann and Panke, 2006; Ro et al., 2006; Stano et al., 2012). The discipline of synthetic biology (SB) has made tremendous advances from the first building of biological parts for use as basic elements to tune every living system (Alper et al., 2005; Gardner et al., 2000; Rosenfeld et al., 2005) to the development of a completely synthetic genome (Gibson et al., 2008) and the most recent achievements in metabolic engineering and protein design (de Lorenzo, 2014; Keasling, 2012; Munnelly, 2013; Salis et al., 2009; Stano et al., 2012). Natural biological processes inspire concepts for effective solutions to an existing problem or for generating novel capabilities (Purnick and Weiss, 2009); the resulting design can be more robust and/or efficient than systems that have evolved naturally (Schmidt and de Lorenzo, 2012). These objectives are arduous to achieve, however, due to the complexity of living organisms (Nielsen et al., 2013). The use of microorganisms (especially bacteria) solves this problem in part and has rendered less complicated the development of promising strategies for a number of applications such as production of drugs or biofuels (Nikel et al., 2014b; Serrano, 2007).

The relative simplicity of prokaryotic relative to eukaryotic systems enabled not only the manipulation of their genomes (this was done from the early discoveries in molecular biology), but also the deconstruction of their biological components, which can be considered metaphorically as electronic parts (Endy, 2005). The "first wave" of synthetic biology (Purnick and Weiss, 2009) focused on remaking, inventing and optimizing genetic devices and small modules. These basic elements, represented by promoters, ribosome binding sites, transcription factors,

terminators and small RNAs, are combined to form modules with specific activities (**Fig. 1**). Examples of modules include oscillators (Elowitz and Leibler, 2000), switches (Gardner et al., 2000), cascades (Hooshangi et al., 2005; Moon et al., 2012), pulse generators (Basu et al., 2004), delayed circuits (Weber et al., 2007), and logic formulas (Silva-Rocha and de Lorenzo, 2011) which can be used to regulate gene expression, protein function, and cell-cell communication (Munnelly, 2013); (Brophy and Voigt, 2014; Michalodimitrakis and Isalan, 2009). One of the most intriguing objectives is to manipulate these basic elements (often derived from different sources) and combine modules to obtain robust functional units. Current efforts focus on characterizing and standardizing parts and small modules by measuring, manipulating and matching their input-output thresholds (see next section) (Lee et al., 2013).

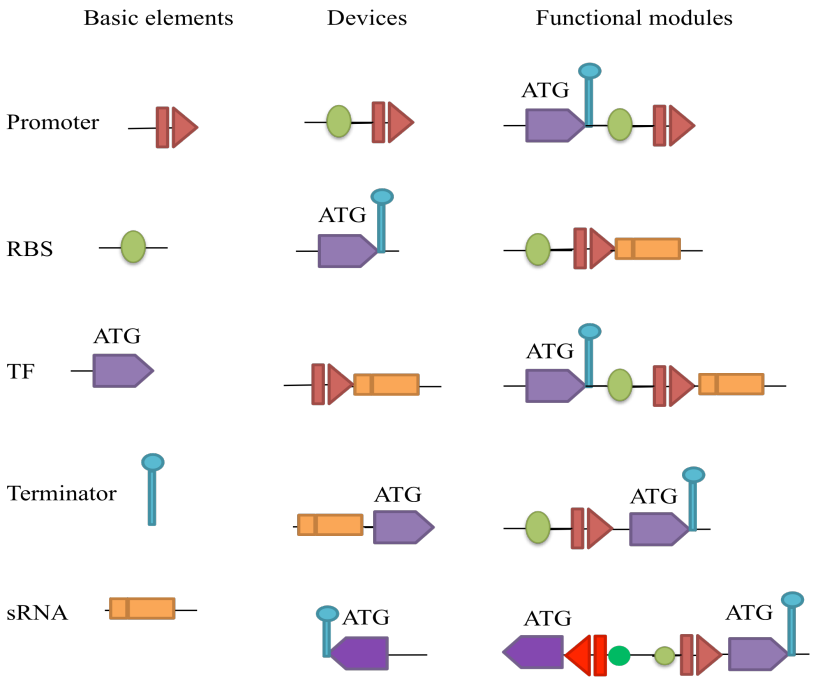

Fig. 1. T**he elements used in synthetic biology.** Basic parts, represented by promoters, ribosome binding sites (RBS), transcription factors (TF), terminators and small RNAs, are combined to form devices, which are assembled to form functional systems.

The following phase ("second wave",(Purnick and Weiss, 2009) of SB consists of integrating genetic parts and modules to create working systems or, if we adopt the

language of engineering, functional circuits. Building synthetic circuits nonetheless remains one of the most challenging aspects of genetic engineering, because even those that we think of as the simplest require deep understanding of regulatory processes and precise tuning in their expression. It is also important to consider that all parts that constitute a circuit appear in genetic contexts that interfere with the environment, and can lead to responses that differ from their original context and are sometimes unpredictable (Endy, 2005).

Nielsen *et al*. reviewed the most recent advances in genetic circuit design and focused on the expansion of orthogonal regulatory parts, defined as genetic parts that do not interfere with each other, such that they can be used together in a circuit (Nielsen et al., 2013). They also proposed new approaches to control expression more precisely, such as more sophisticated computational operations and the complete organization of parts libraries (Brophy and Voigt, 2014; Medema et al., 2012; Nielsen et al., 2013). As is true for many emerging research fields, achieving the full potential of SB applications will require continued development of technologies that enable acquisition of new research tools (Arkin, 2008; Kitney and Freemont, 2012). Because of the interdisciplinarity of this field –which includes biology, engineering, mathematics and informatics–, these technologies cover a broad range of possibilities, depending on the type of research (Kahl and Endy, 2013) .The core of technical areas includes standardization of biological parts, DNA synthesis and assembly (Casini et al., 2014; Chao et al., 2014; Wang et al., 2012)sequencing (Anderson and Schrijver, 2010) and genome editing (Nikel et al., 2014b). In the next section, we will focus on the importance of standardized genetic tools as means to generate and assemble functional modular components, to achieve a better understanding of life processes, and to develop new applications.

1.2 Standard tools in synthetic biology

Every engineering field has a set of standards used to design systems, devices and parts Synthetic biology similarly aims to apply these principles of abstraction and characterization to facilitate engineering of new biological systems, constituted of

DNA-encoded components (Canton et al., 2008; de Lorenzo and Danchin, 2008; Ellis et al., 2011; Porcar et al., 2011). These "parts", such as promoters or open reading frames (ORF), can be considered separately or can be combined to form functional devices that give a predictable response –for example, a new pathway that provokes expression of a specific protein in a host cell. This approach is defined as forward engineering and consists of rational design of complex objects with properties that can be predicted quantitatively from the attributes of their components (Munnelly, 2013; Nikel et al., 2013c). The possibility of using several biological parts, often engineered in diverse laboratories, can help to build more complex biological systems. It is thus very important for scientific communities to communicate and share these components, and in this way, the use of standard tools can provide enormous help (Henkel and Maurer, 2009).

To be defined as standard, a tool must satisfy the following requirements: 1) results obtained must be comparable between different samples and laboratories, 2) experiments must be reproducible in other laboratories, 3) researchers must use same language or nomenclature, and 4) resulting data must conform to electronic data processing (Muller and Arndt, 2012). The BioBricks Foundation (http://biobricks.org/), begun in 1999, was a fundamental advance for organization and standardization of synthetic biology; this platform collects a number of modular biological parts engineered by undergraduate students that participate in the iGEM competition (Smolke, 2009). These DNA units (BioBrick DNA) have standardized flanking sequences that enable assembly by a simple restriction/ligation method (Pasotti et al., 2012; Shetty et al., 2008) (**Fig. 2**). All BioBrick DNAs are stored in the public access Registry for Standard Biological Parts (http://partsregistry.org), which permits easier sharing and immediate use. A report in 2013 (Munnelly, 2013) stated that the registry housed more than 7,000 available parts, which indicates the rapid increase in biological components produced (Vilanova and Porcar, 2014).

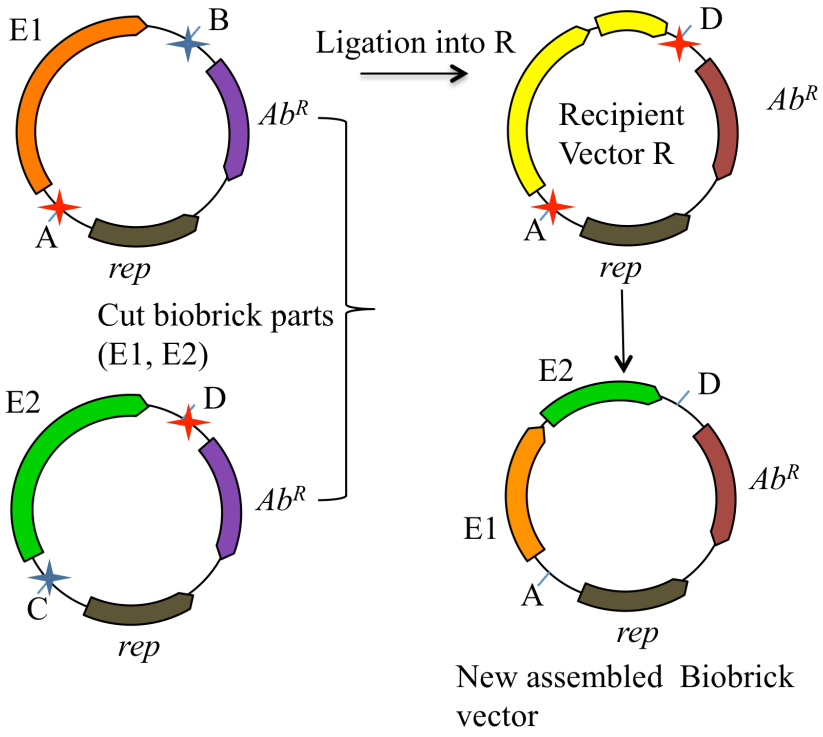

Fig. 2. **Biobrick assembly strategy.** Biobrick DNA units are flanked by restriction sites sequence (A, B, C, D) that enable assembly in a third Biobrick vector through a traditional digestion and ligation method. Briefly, the prefix part E1 is digested by enzymes A and B, while suffix part E2 is digested by enzymes C and D. Vector R is the recipient in which two parts are assembled with restriction enzymes A and D. Three fragments are ligated and transferred to *E. coli* cells by transformation. Positive colonies are selected in LB (Luria-Bertani) media supplemented with specific antibiotics.

The pSEVA database (SEVA-DB, http://seva.cnb.csic.es ref) was recently proposed as an innovative platform of molecular tools to create a set of formatted vector plasmids that can be used widely in bacteria (Silva-Rocha et al., 2013), and that are compatible with old and new cloning and DNA assembly methods. Such vectors are available in the SEVA-DB and have a synthetic, modular and interchangeable structure. These modules include housekeeping elements such as broad host range origin of replication and antibiotic markers, as well as cargos such as expression systems and reporter genes punctuated by terminator sites (**Fig. 3**). The principal advantage of these tools is the possibility to assemble different modules by a standard method using restriction sites that delimit each block. The SEVA format

calls for designation of each vector with a specific code by which all vectors are named pSEVA, followed by a multi-digit cipher (Durante-Rodriguez et al., 2014; Silva-Rocha et al., 2013). Similar to the BioBricks platform, new constructs enter SEVA-DB and are made available as soon as they are verified to meet the standard. The community is encouraged to contribute to this effort to enrich the SEVA collection. The last standardization proposal, for representation of synthetic biology design, is termed SBOL (Synthetic Biology Open Language, http://www.sbolstandard.org/); SBOL is a "core data model" for specification of DNA-level designs (Galdzicki et al., 2014).. The SBOL core defines biological building blocks such as DNA components and enables their hierarchical composition, allowing determination of the substructure and lineage of each design component. The SBOL core also offers a "collection" of data structure to group DNA components in software libraries and catalogs. Given the rapid evolution of synthetic biology, this platform could be useful for information exchange between several different software tools and repositories from industrial and academic partners.

As mentioned above, the vast majority of genetic engineering work is done in bacteria, particularly in *Escherichia coli* (Benedetti I.M., 2012; Nikel et al., 2014b). This is an excellent host for physical assembly of DNA parts, but the large-scale deployment of synthetic biology-derived activities requires a choice of available host. In the next section, we will tackle another issue in synthetic biology, the choice of an appropriate host.

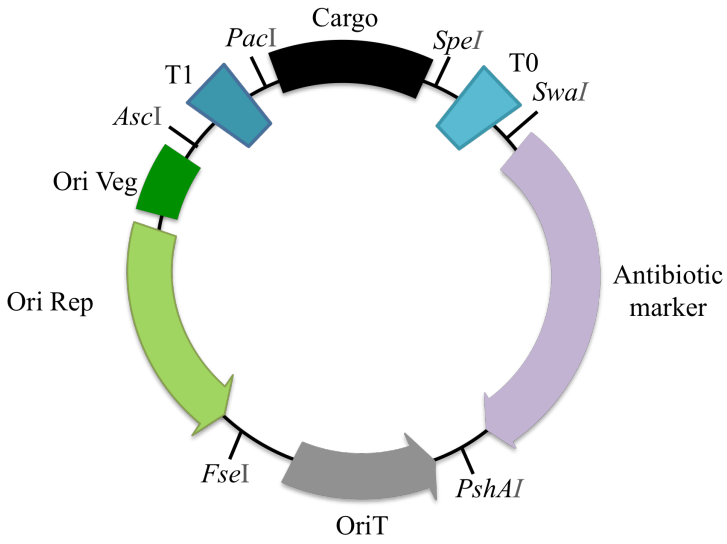

Fig. 3. Overall organization of pSEVA plasmids. pSEVA vectors are formed by three variable modules: cargo (black), a replication origin (green) and an antibiotic marker (purple). Enzymes used to change the functional DNA segments are shown. Modules are separated by three permanent regions shared by all vectors, the T0 and T1 transcription terminators and the *oriT* conjugation origin.

1.3 Chassis for synthetic biology tasks

1.3.1 Where do DNA components assemble? Genetic engineering techniques traditionally used microorganisms as hosts for introduction or manipulation of DNA. Bacteria in particular represented the ideal system, because of their great versatility for genome editing and less complexity compared to eukaryotic organisms (Michalodimitrakis and Isalan, 2009; Nikel et al., 2014b) . Synthetic biology also relies on bacteria as dynamic scaffolds for implanting designed genetic circuits and obtaining new functions. In contrast to traditional genetic engineering, SB involves the design of objects whose function is predicted based on their features; it also needs an autonomous genetic and/or biochemical platform (chassis) for implanting forward-designed biological devices (Purnick and Weiss, 2009; Silva-Rocha and de Lorenzo, 2010). In this way, a microbial chassis adapted for synthetic biology should be derived from bacteria endowed with several metabolic and physiological properties that are amenable to stable genetic reprogramming (Nikel et al., 2014b). An optimal bacterial chassis requires a streamlined genome

that encodes only basic biological functions for self-maintenance, growth and stress resistance; elements such as other cell structures are usually removed as they might not be needed in biotechnology settings (Martinez-Garcia et al., 2014b). As an example of an ideal chassis, *E. coli* has been and continues to be used as a reference strain for recombinant DNA techniques and consequently, for SB applications.

1.3.2 *Pseudomonas putida*, an alternative host for synthetic biology. Although *E. coli* is an optimal experimental model for testing new functions, it is not appropriate for some objectives that synthetic biology proposes. Some of these include production of complex biofuels or the biodegradation of recalcitrant compounds, which often proceed by accumulating intermediates toxic for *E. coli* strains (Schaechter and View From Here, 2001). It is thus opportune to consider different available hosts; soil bacteria are a good model, given their great adaptability in an environment that is continuously changing (Gurney and Thomas, 2011). Several species of the genus *Pseudomonas* appear to fulfill many of the conditions required of an optimal platform strain. *Pseudomonas* species are Gram-negative aerobic gamma-proteobacteria and colonize a wide variety of niches such as soil, the rizosphere, waters, and the human body (Loper et al., 2012; Palleroni, 2010). In addition to this great adaptability, which includes resistance to exogenous and endogenous stress, *Pseudomonas* species are able to produce many bioactive compounds, including antibiotics (Chen et al., 2013).

Not all *Pseudomonas* can be used as platform strains, however; some species produce virulence factors and are plant or human pathogens. One example is *Pseudomonas aeruginosa*, which synthesizes a viscous compound that constitutes the exopolysaccharide matrix the first factor that infects lungs of cystic fibrosis patients (Balasubramanian et al., 2013; May and Chakrabarty, 1994). Another example is *Pseudomonas syringae*, which infects plant surfaces or fruits (such as tomatoes) and like *P. aeruginosa*, uses exopolysaccharides or other attachment elements as virulence factors (Xin and He, 2013).

The most attractive branch of the Pseudomonadacae family is the non-pathogenic species *P. putida,* a rapidly growing bacterium isolated from most temperate waters and soils (especially from contaminated soils). It is a nutritional opportunist and a metabolically versatile microorganism that can mineralize a broad range of organic wastes such as aromatic hydrocarbons, chloro and nitro organic compounds, pesticides, herbicides and even explosive chemicals (Fernandez et al., 2009; Garmendia et al., 2008; Nikel et al., 2014a; Nikel et al., 2013b; Timmis, 2002). *P. putida* strains are often resistant to antibiotics, disinfectants, detergents and heavy metals (Chen et al., 2013; Paez-Espino et al., 2014),(Nogales et al., 2008; Velazquez et al., 2006) and can develop resistance to organic solvents thanks to an efficient system of efflux pumps that can extrude molecules such as toluene (Ramos et al., 1995; Rojas et al., 2003). The ability to degrade or tolerate a wide spectrum of compounds is also due in part to multiple plasmids and transposons that code for specific catabolic pathways (Kado, 1998; Yano et al., 2010). Well-studied examples of catabolic genetic elements are the TOL plasmid *pWW0*, which encodes toluene and xylene catabolism, and the NAH7 plasmid (Schell and Poser, 1989), which controls expression of a naphthalene degradation pathway. A final feature of biotechnological interest that characterizes *P. putida* is accumulation of intracellular polyester granules as storage material (La Rosa et al., 2014; Nikel et al., 2013a). This material is particularly interesting because of its plastic properties, which makes it a possible candidate to replace oil-derived plastics in several types of packaging. It is thus not surprising that *P. putida* is considered a promising candidate for development of a safe strain for recombinant DNA techniques and environmental applications.

1.3.3 *Pseudomonas putida* KT2440. The most relevant *P. putida* strain is *P. putida* KT2440 (Regenhardt et al., 2002), a derivative of the toluene-degrading wild type *Pseudomonas* mt-2 bacterium , which harbors the TOL plasmid *pWW0* (Worsey and Williams, 1975). *P. putida* KT2440 is a plasmid-free derivative of *P. putida* mt-2 and was certified in 1982 by the Recombinant DNA Advisory Committee (RAC) of the U.S. National Institutes of Health as the host strain for the first host-

vector biosafety (HV1) system for gene cloning in Gram-negative soil bacteria (Federal Register, 1982) (Timmis, 2002). *P. putida* KT2440 shares interesting traits with other *P. putida* strains (growth on a broad range of recalcitrant compounds, tolerance to solvents, antibiotics and heavy metals, high reducing power) and for this reason it has been exploited as a host for a variety of genetic tools such as mini-transposons developed to analyze, clone and manipulate genes from other soil bacteria (Arce-Rodriguez et al., 2012; Chavarria et al., 2013; Milanesio et al., 2011).

To consider this strain an optimal chassis for synthetic biology applications, however, it is necessary manipulate its genome to enhance advantageous features and eliminate undesirable traits. A number of genetic tools have been optimized to edit the *P. putida* KT2440 genome, resulting in multiple deletions of several genes involved in motility (Martinez-Garcia et al., 2014b) and phage-related functions (Martinez-Garcia et al., 2014a), among others. A series of vectors and plasmids were engineered to insert genes in *P. putida* KT2440; the most prominent example is the SEVA platform (see previous section), which includes a large collection of standardized plasmid vectors (Nikel and de Lorenzo, 2013a, b)recently described formatted vectors that can be used to engineer a *P. putida* KT2440 able to degrade 1,3-dichloroprop-1-ene in conditions of limited oxygen supply.

Not only are plasmids and vectors used to insert heterologous DNA, however; sometimes these parts must be integrated into the chromosome. One possible solution is the use of strategies based on Tn*5*, Tn*10* and Tn*7* mini-transposon vectors, which can deliver a desired DNA fragment to the *P. putida* genome as part of a hybrid mobile element (Martinez-Garcia and de Lorenzo, 2012). The advantages of this approach are not only the generation of stable chromosome insertions, but also the possibility of using different transposition mechanisms (random or site-specific). *P. putida* KT2440 is rich in regulatory elements that respond to a diverse repertoire of chemical effectors. Some are transcription factors activated by xylenes (XylR (Fraile et al., 2001), *m*-toluate (XylS (Kessler et al.,

1994a), salicylate (NahR (Schell and Poser, 1989) and short-chain alkanes (AlkS (Cases and de Lorenzo, 2005; Rojo, 2010); they can be assembled with an output promoter and also act as alternative expression systems that can be used as orthogonal devices (**Fig. 4**). Such manipulations are not restricted to regulator-promoter pairs, but also enable new input to transcription factors themselves, as well as to housekeeping proteins by engineering protease-cleaving sites in their structure, thereby modifying their behavior (Calles and de Lorenzo, 2013).

P. putida strains are distributed ubiquitously in the soil and usually form biofilms on surfaces such as roots or other soil elements. *P. putida* KT2440 is able to form biofilms on abiotic surfaces, the shape of which is determined by the physiological condition of cells, nutrient accessibility, and surface chemistry, although these films are not robust (D'Alvise et al., 2010). This suggests the possibility of engineering *P. putida* KT2440 to form biofilms with catalytic activities. One example was described in a study in which they engineered a recombinant Pseudomonas whose biofilms produce fine chemicals such as *n*-octanol and (S) styrene oxide (Halan et al., 2011); another is the engineering of *P. putida* KT2440 as a catalytic biofilm, to be described in this thesis.

1.4 Dynamics of genetic networks: analysis of individual heterogeneity in microbial populations

To optimize the engineering of microbial strains with new abilities, it is necessary to understand their behavior in natural contexts. In this section, we describe the most prominent approaches to the study of variability in single microbial cells, to understand their regulatory properties.

Naturally occurring bacteria most often exist in populations in which spontaneous mutations, coupled with different types of selection pressure and environmental changes, result in rapid genetic diversification (Rainey and Travisano, 1998). Exploration of new niches via bet-hedging mechanisms usually facilitates adaptation to local environmental fluctuations (Veening et al., 2008). Alternative

traits can nonetheless be manifested simultaneously in distinct fractions of genetically identical populations, thereby originating phenotypic bifurcations. Increasing evidence suggests that the ability to generate phenotypically diverse individuals is an important evolutionary strategy for enduring changing environments (Rainey et al., 2011). Stochastic, regulatory and mutational changes can contribute to overall population fitness, sometimes at the cost of cell robustness (Levy and Siegal, 2012).

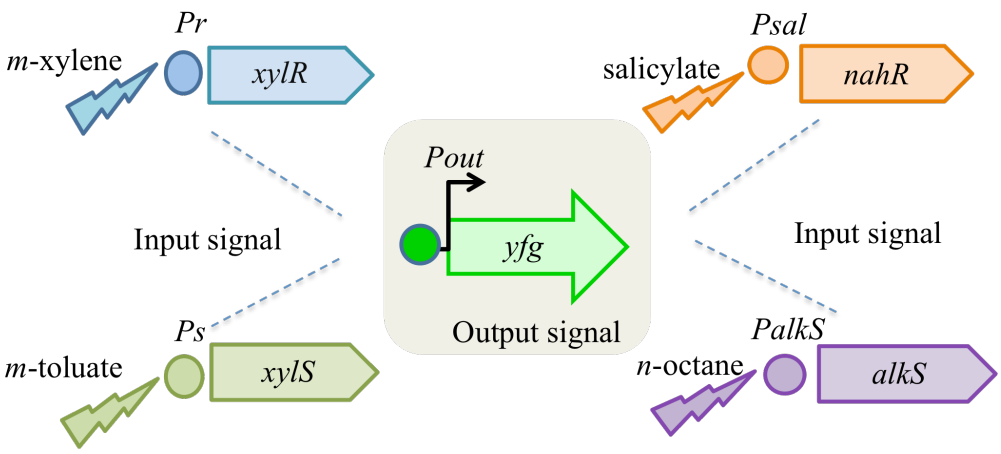

Fig. 4. Transcriptional factors encoded by *Pseudomonas putida*. These regulatory factors are activated by compounds such as xylenes (XylR), *m*-toluate (XylS), salicylate (NahR) and short-chain alkanes (AlkS); they can be assembled with an output promoter and serve as alternative expression systems that can be used as orthogonal devices.

Programmed phenotypic bifurcations and specializations have long been known in bacteria (Hadden and Nester, 1968); more recently, metabolic diversification in individual cells has been documented in strains such as *E. coli* and *P. putida* (Ackermann, 2013). The phenotypic variation reflects a normal circumstance in nature, which can provoke serious consequences in cases such as clinical settings (presence of antibiotic-resistant bacteria, (Balaban et al., 2004), biotransformation processes (generation of non-productive variants) or bioremediation strategies (metabolic diversification of pollutants that degrade bacteria). Because these features are borne by individual cells, they are often detected by standard

population-level measurements of gene expression (Lidstrom and Konopka, 2010), particularly in laboratory settings. This makes the availability of appropriate tools for single cell analysis crucial for identifying the phenomena and understanding their molecular basis.

1.4.1 Noise, bimodality, and bi-stability. Every promoter is subjected to stochastic fluctuations caused by *intrinsic* and *extrinsic* molecular noise (Silva-Rocha and de Lorenzo, 2010). These two types of noise may be experimentally exposed and analyzed by studying signals originating from the same promoter when fused with two reporters. Under some circumstances (e.g. as caused by the dearth of one limiting component of the transcription initiation machinery), stochasticity originates as a bi-modal behavior in which, upon the appearance of a given signal, the transcriptional state of a specific regulatory device splits into two phenotypically different cell types: one where the expression adopts an ON state and another where the same bacteria take an OFF values for the same promoter. If these states are maintained even after the trigger input is gone, then the expression device qualifies as *bi-stable system*. This indicates that once a transcriptional regime is reached, it will be maintained after the disappearance of the actual signal, which elicited the bifurcation of the ON and OFF states. Conversely, in graded behavior, all cells in the population switch to ON state (promoter is activated) upon sensing the cognate signal (Becskei et al., 2001). In this system, the observed change in the expression level at the population scale faithfully reflects the accumulation of the transcript-reporter in individual cells (Kaern et al., 2005; Keasling, 2012).

1.4.2 Analysis of transcriptional fluctuations in single cells. Reporter gene fusions are essential tools for the study of gene regulation and in exposing the stochastic nature of some gene regulatory networks. These fusions (involving the promoter of interest and reporter genes that deliver an optical output) have been used to explore regulatory elements that control gene expression (de Las Heras et al., 2010; Zaslaver et al., 2006). A variety of reporter genes is currently available

for these studies; those that encode β-galactosidase and luciferase are the most frequently used as enzyme reporters and that for green fluorescent protein (GFP) for numerous applications in living cells as a non-invasive marker. The products of the *lux* genes and *lacZ* have some inherent limitations, however, due to the need for indirect measurement (Silva-Rocha and De Lorenzo, 2012a) or a strong dependence on the metabolic state of the cells (Meighen, 1991). In contrast, the advantage of fluorescent proteins is that they do not need a specific substrate to be activated and they are useful descriptors for monitoring stochastic processes at the single cell level (Benedetti I.M., 2012; Chalfie et al., 1994; Cox et al., 2010; Suarez et al., 1997; Young et al., 2011).

A number of currently available systems are based on plasmid (multicopy) or transposon (single copy) vectors; these elements are used as a platform to deliver transcriptional fusions that usually include a regulatory element (a promoter) as the input and a reporter gene (e.g., *gfp*) as the element that provides the output signal (Benedetti I.M., 2012; Schweizer, 2001; van der Meer and Belkin, 2010). Another approach to analysis of cell heterogeneity is based on the use of transposon vectors to produce single insertions of the reporter systems into the chromosome of target bacteria (Choi and Kim, 2009; Damron et al., 2013; de Las Heras et al., 2012; de Lorenzo et al., 1990). The obvious advantage of this method is that it avoids variability and other detrimental effects caused by multi-copy vectors (Cox et al., 2007). The vectors used most often for such endeavors in Gram-negative bacteria are based on mini-Tn*5*, mini-Tn*10* and mini-Tn*7* transposons. These elements yield single insertions into the chromosome, randomly (Choi and Kim, 2009; Choi and Schweizer, 2006; de Lorenzo et al., 1990; Reznikoff, 2008) or at a specific site (Tn*7* only (Choi et al., 2005; Schweizer, 2001). A number of tools have been developed with these functions, and in some cases reporters were combined in a bi-cistronic operon to obtain a dual system that allows simultaneous assessment of transcription activity in a population and in single cells (Benedetti and de Lorenzo, 2014), , (Silva-Rocha and de Lorenzo, 2014).

1.4.3 Common techniques to detect output reporter signal. A number of high-throughput techniques permit quantification of the signal emitted by fluorescent reporters such as GFP, which are fundamental for analysis of single cell activity. The most commonly used methods are described in the following subsections.

Time-lapse microscopy

Tracing the manifestation of a given phenotype of individual cells in time and space has been instrumental to understanding bi-stability phenomena. One of the first and most useful methods is time-lapse microscopy (Rosenfeld et al., 2005), which combines quantitative image analysis and fluorescent protein reporters for direct inspection of single cells over time. In a notable case, Ackermann and colleagues (2003) adopted this approach to determine how lineage and history can affect cell-to-cell variability in the ageing and senescence of *Caulobacter crescentus*. Automated lineage tracking of individual cells through several generations showed that even an apparently symmetrically dividing microorganism such as *E. coli* does age (Ackermann et al., 2003). GFP fusions used with time-lapse microscopy exposed genes whose transcription results in high levels of phenotypic noise, e.g., in *Salmonella* (Freed et al., 2008). In this case, expression of flagellar genes turned out to be the noisiest, implying a possible role for phenotypic noise in pathogenesis. The range of applications of microscopy approaches can be enhanced by techniques that also allow separation of individual cells from an entire population, as described in the next paragraph.

Multi-parameter flow cytometry

The use of fluorescent reporters followed by direct single cell measurement of activity via microscopy or flow cytometry (FC) permits evaluation of population heterogeneity specifically (Bergquist et al., 2009; Brehm-Stecher and Johnson, 2004; Czechowska et al., 2008), a large number of cells in suspension is readily analyzed by FC (Shapiro, 2005). FC permits multi-parameter analysis of several thousands of cells per second and their real-time quantification in the form of an electronic signal as the particles pass an excitation source, which provides

information on their size, complexity and fluorescence. Within the last few years, FC has assumed crucial importance for the characterization of regulatory networks that define genetic cell-cell variability (Brehm-Stecher and Johnson, 2004; Unge et al., 1999). In a typical FC study, an isogenic population bearing the transcriptional fusion of a given promoter to *gfp* can appear as a unique distribution (usually represented as histograms or two-dimensional dot plots; (Bergquist et al., 2009) or split in two (or more) subpopulations. In the first case (unimodal distribution), the promoter shows a typical ON/OFF state, while in the second (bimodal distribution), the distinct GFP intensities within the same population show that the promoter can be activated in different time frames, generating heterogeneity at the single cell level (Nikel et al., 2013c) (**Fig. 5**). The many recent applications of FC show that this approach is indispensable for describing how a biological system is subject to fluctuations that determine its physiological state.

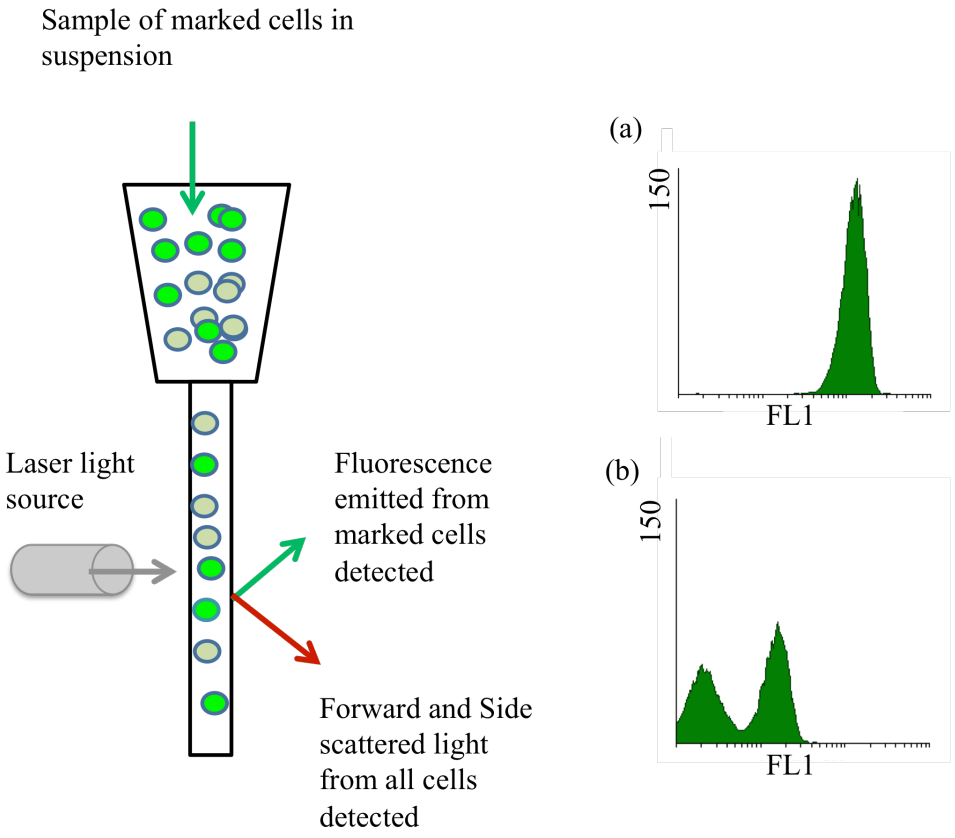

Fig. 5. **Flow cytometry assay and data representation**. In a typical FC setup, particles/cells passing through the beam will scatter light, which is detected as forward scatter (FS) and side scatter (SS). The combination of scattered and fluorescent light is detected, collected and analyzed. Whereas FS correlates with cell size, SS depends on particle/cell density (such as number of cytoplasmic granules, membrane size and complexity). In this way, cell populations can often be distinguished based on differences in their size and density/complexity. Fluorochromes used for detection in FC emit light when excited by a laser at the excitation wavelength of the molecule being tested. These fluorescent-stained particles or cells can be detected individually and the data analyzed. In the case of unimodal distribution **(a)**, the cells marked show a typical ON/OFF distribution, while in the bimodal distribution **(b)**, the different fluorescence intensities within the same population indicate that the cells emit signals in different time frames, generating heterogeneity at the single cell level.

5. Issues and aims

Synthetic biology is an emerging discipline that has evolved to engineer living organisms by focusing on their specific abilities. The main feature of a neo-

engineered organism is the presence of synthetic devices constituted of standardized parts. Standardization is an important issue in the SB community, and responds to the need to share DNA parts, modules and molecular tools that every scientist can adopt and use. We have several useful platforms for finding and combining standardized tools: the BioBricks registry of synthetic parts, the pSEVA plasmid collection and the more recent SBOL open-source standard are the most prominent examples.

The vast majority of genetic engineering studies are done in bacteria, particularly in *Escherichia coli*. Other microorganisms can nonetheless be considered as hosts for new devices; one of these is *Pseudomonas putida*, specifically, the strain *P. putida* KT2440. This Gram-negative soil bacterium colonizes roots, is extremely resistant to many toxic solvents, and is able to use many of these as an energy source. Although the molecular mechanisms and physiology of this bacterium have been explored for many years, some questions remain unanswered regarding regulatory dynamics and biotechnological application. For this reason, we propose a collection of standardized genetic tools for *P. putida* that will be instrumental to clarify some of these features.

II. OBJECTIVES

General objective:

The principal objective of this work was the design of functional and standardized molecular tools to be used to comprehend the regulatory and biochemical properties of *P. putida* KT2440.

Specific objectives:

1) To engineer and validate standardized genetic tools based on plasmid and transposon mechanisms for analysis of gene expression in Gram-negative bacteria.
2) To explore the dynamics of the XylR-*Pu* regulatory network, which in *P. putida* is specialized in degrading aromatic compounds.
3) To analyze XylR production *in vivo* after Crc protein-mediated repression.
4) To enhance *P. putida* KT2440 ability to form a catalytically active biofilm.

III. MATERIAL AND METHODS

1. Culture conditions and media

Escherichia coli strains were grown at 37ºC in Luria-Bertani (LB) medium or in M9 minimal medium supplemented with 0.4% (w/v) glucose or succinate, 0.1% (w/v) casamino acids (except for BW25113 strain), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.05% (w/v) vitamin B1 (Benedetti I.M., 2012). *Pseudomonas putida* cells were incubated at 30ºC in M9 minimal medium supplemented with 2 mM $MgSO_4$ and 25 mM succinate, citrate or glucose as the sole carbon source (Abril et al., 1989). When required, kanamycin (Km, 50 μg mL^{-1}), streptomycin (Sm, 50 μg mL^{-1}), gentamycin (Gm, 10 μg mL^{-1}), ampicillin (Ap, 150 μg mL^{-1}), tetracycline (Tc, 10 μg mL^{-1}) or chloramphenicol (Cm, 30 μg mL^{-1}) was added to the growth media. For the haloalkane dehalogenase assays, cells were grown in low-chloride minimal medium containing the same salts as M9, but with with $(NH_4)_2SO_4$ instead of NH_4Cl and Na_2SO_4 instead of NaCl. All inducers indicated were purchased from Sigma-Aldrich, while flow cytometry materials (buffers, calibration beads) were purchased from Milteny Biotec.

2. Bacterial strains

The strains used in this study are listed in Table 1. *E. coli* CC118 or its variant *E. coli* CC118*λpir* (Herrero et al., 1990) were used as the host for the plasmid constructs. *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969) (pRK600) was used as a helper for triparental mating, as described (Keen et al., 1988). *E. coli* MG1655 (Guyer et al., 1981) was used as the host for the *PlexA* → GFP-*luxCDABE* construct and *E.coli* BW25113 (Baba et al., 2006) was used as host strain for pSEVA2312-nox and pSEVA234-nox. *Escherichia coli* DH5α *λpir* was used as host for the suicide plasmid pTNS-1 bearing the transposase complex (Choi et al., 2005). Wild-type strain *Pseudomonas putida* mt-2 (Worsey and Williams, 1975) and TOL-less variant *P. putida* KT2440 (Bagdasarian et al., 1981) were used as recipient strains for mating experiments. *P. putida* KT2440 Δall-Φ (Martinez-Garcia et al., 2014a) was used as recipient strain for pSEVA2311 and pS-eDQ for biofilm analysis by fluorescence microscopy.

Table 1. Description of bacterial strains used in this study

Strain	Description	Reference
P. putida strains		
mt-2	*P. putida* wild type with pWW0 plasmid	Worsey and Williams, 1975
KT2440	Wild-type strain derived of *P. putida* mt-2 cured of the pWW0 plasmid	Bagdasarian et al., 1981
mmt-2 *Pu*	Sm^R; *P. putida* MEG3 derivative harbouring a *Pu→GFP–lacZ* fusion in the chromosome	Silva-Rocha and de Lorenzo, 2012b
KT2440 *Pu*-GFP	Km^R, *P. putida* KT2440 inserted with mini-Tn*5 Pu→GFP* transcriptional fusion	Lab collection
KT-IB1	GmR, *P. putida* KT2440 inserted with mini-Tn*7 lacI*^{q}*Ptrc→xylR* transcriptional fusion	This study
KT2442 Crc^-	Tc^R, *crc* null variant of KT2440	Moreno and Rojo, 2008
KT2440 BG	Gm^R, *P. putida* KT2440 inserted with mini-Tn*7* BCD2-msfGFP	This study
KT2440 BG- *PEM7*	Gm^R, *P. putida* KT2440 inserted with mini-Tn*7 PEM7* XylR →BCD2-msfGFP transcriptional fusion	This study
KT2440 BGS	Gm^R, *P. putida* KT2440 inserted with mini-Tn*7* XylS/*Pm*→BCD2-msfGFP transcriptional fusion	This study
mt-2 BG-*Pm*	Gm^R, *P. putida* KT2440 inserted with mini-Tn*7 Pm*→BCD2-msfGFP transcriptional fusion	This study

mt-2 BG-*Pm*	Gm^R, *P. putida* KT2440 inserted with mini-Tn*7* *Pm*→BCD2-msfGFP transcriptional fusion	This study
KT2440 SynPro(s) (Collection)	Gm^R, *P. putida* KT2440 inserted with mini-Tn*7* synthetic promoters *(SynPro)* →BCD2-msfGFP transcriptional fusions	This study
KT-R1a	*P. putida* KT2440 derivative, Gm^R, msfGFP translational fusion to *Pr*-XylR	This study
KT-R1b	*P. putida* KT2440 derivative, Gm^R, msfGFP translational fusion to *PEM7*-XylR	This study
KTC-R1a	*P. putida* KT2442 Crc^- derivative, Gm^R, msfGFP translational fusion to *Pr*-XylR	This study
KTC-R1b	*P. putida* KT2442 Crc^- derivative,.Gm^R, msfGFP translational fusion to *PEM7* XylR	This study
KT- R2a	*P. putida* KT2440 derivative, Gm^R, msfGFP translational fusion to *Pr*-XylR mutated in *crc* site	This study
KT-R2b	*P. putida* KT2440 derivative, Gm^R, msfGFP translational fusion to *PEM7*-XylR mutated in *crc* site	This study
KTC-R2a	*P. putida* KT2442 Crc^- derivative,.Gm^R, msfGFP translational fusion to *Pr*-XylR mutated in *crc* site	This study
KTC-R2b	*P. putida* KT2442 Crc^- derivative,.Gm^R, msfGFP translational fusion to *PEM7*-XylR mutated in *crc* site	This study
mt-2 *Pu*-GFP	Gm^R, *mt-2* inserted with mini-Tn*7* *Pu*→GFP transcriptional fusion	This study

KT2440 Δall-Φ	*P. putida* KT2440 derivative with prophage 1, prophage 4, prophage 3, and prophage 2 deleted	Martinez-Garcia et al., 2014a
E. coli strains		
HB101	Sm^R, *hsdR*$^-$*M*$^+$, *pro, leu, thi, recA*	Boyer and Roulland-Dussoix, 1969
CC118	F-, Δ (*ara-leu*) 7697, *araD*139, Δ (*lac*)X74, *pho*AΔ20, *ale*, *galK*, *thi*, *rpsE*, *rpoB*	Herrero et al., 1990
MG1655	Prototrophic; *recA*$^+$, reference K12 strain	Guyer et al., 1981
CC118λpir	λ pir phage lysogen of CC118	Herrero et al., 1990
DH5αλpir	λ pir phage lysogen of DH5α	Lab collection
BW25113	*F*$^-$ Δ λ$^-$ (*araD-araB*)567 Δ*lac*Z4787(::*rrnB*-3) *rph*-1 Δ (*rhaD-rhaB*)568 *hsdR*514	Baba et al., 2006

3. Plasmids

The plasmids used in this study are described in **Table 2**. Procedures of specific plasmid construction will be described in the following sections.

Table 2. Plasmids used in this study

Plasmid	**Description**	**References**
pRK600	Cm^R, *oriV* ColE1, *tra*+*mob*+ of RK2, helper plasmid for mobilization in tripartite conjugations	Keen et al., 1988
pTnS-1	Ap^R, *RK6* replicon encodes the *TnSABC + D* specific transposition pathway	Choi et al., 2005

pSEVA221	Km^R, *oriV* RK2*, oriT*; standard broad-host range plasmid for Gram-negative bacteria	Silva-Rocha et al., 2013
pSEVA224	Km^R, *oriV* RK2*, oriT*; standard broad-host-range *lacI*^{q}*/Ptrc* expression system	Silva-Rocha et al., 2013
pSEVA236	Km^R, *oriV* pBBR1*, oriT*; standard broad host-range with *luxCDABE* reporter system	Silva-Rocha et al., 2013
pGreenTIR	Ap^R, *oriV* ColE1; promoterless cloning vector with *gfp tir* gene	Miller and Lindow, 1997
pGLR1	Km^R, *oriV* pBBR1*, oriT*; pSEVA236-derivative with dual GFP-*luxCDABE* reporter system	Benedetti and de Lorenzo, 2014
pGLR2	Km^R, *oriV* RK2*, oriT*; pSEVA221-derivative with dual GFP-*luxCDABE* reporter system	Benedetti and de Lorenzo, 2014
pGLR1-*Ptrc*	Km^R, *oriV* pBBR1*, oriT*; pGLR1-derivative with LacI^q*/Ptrc* expression system cloned as a *Pac*I/*Avr*II fragment	Benedetti and de Lorenzo, 2014
pSEVA226	Km^R, *oriV* RK2 , standard broad-host-range with *luxCDABE* reporter plasmid	Silva-Rocha et al., 2013
pSEVA211	Km^R, *oriV* R6K, *oriT*; standard broad-host-range plasmid for Gram-negative bacteria	Silva-Rocha et al., 2013
pMK	Km^R, Gm^R, *oriV* ColE1, leading miniTn*7* as *Asc*I-*Swa*I cassette	GeneArt

pTn7-M	$Km^R Gm^R$, *oriV* R6K, *oriT*; standard mini-Tn*7* with MCS	Lab collection
pVTRA-XylR	Cm^R, pSC101 replicon-based vectors with excisable expression of $LacI^q$/*Ptrc*-XylR *Not*I cassette	Perez-Martin and de Lorenzo, 1996
pIB1	Gm^R, Km^R, *oriV* R6K, *oriT*; pTn7-M with $LacI^q$/*Plac-xylR* cloned as *Not*I fragment	This study
pSEVA2513	Km^R, *oriV* RSF1010, *oriT*; standard broad host range with *PEM7* promoter	Lab collection
pSEVA228	Km^R, *oriV* RK2, *oriT*; standard broad-host range with XylS/*Pm* expression system	Silva-Rocha et al., 2013
pTn*7*-BG	Gm^R, Km^R, *oriV* R6K, *oriT*; pTn*7*-M with translational coupler BCD2 and msfGFP cloned as *Sac*I/*Bam*HI fragment	This study
pTn*7*-BG-*PEM7*	Gm^R, Km^R, *oriV* R6K, *oriT*; pTn7-BG derivative with *PEM7* cloned as *Pac*I/*Avr*II fragment	This study
pTn*7*-*Pm*	Gm^R, Km^R, *oriV* R6K , *oriT*; pTn*7*-BG derivative with *Pm* cloned as *Pac*I/*Avr*II fragment	This study
pTn*7*-BGS	Gm^R, Km^R, *oriR6K*, *oriT*; pTn*7*-BG derivative with XylS/*Pm* cloned as	This study

	PacI/*Avr*II fragment	
pTn*7*-SynPro (Collection)	Gm^R,Km^R, *oriV* R6K , *oriT*; pTn*7*-BG derivatives with synthetic promoters cloned as *Pac*I/*Avr*II fragments	This study
pTn*7*-*Pu*GFP	Gm^R, Km^R, *oriV* R6K , *oriT*; pTn*7*-M derivative with *Pu*→GFP transcriptional fusion	This study
pSEVA231	Km^R, *oriV* pBBR1, *oriT*; standard broad-host-range	Silva-Rocha et al., 2013
pSEVA2311	Km^R, *oriV* pBBR1, *oriT*; standard broad-host-range with *chnR/PchnB* expression system	Lab collection
pGFP-UV-DB3	Ap^R, *oriV* ColE1, pUC with GFP	Kemp et al., 2013
pMLK1	Ap^R, *oriV* ColE1, pET28a carrying *CpRK*200S (*cprK1*) of *D. hafniense*	Kemp et al., 2013
pSEVA2312	Km^R, *oriV* pBBR1, *oriT*; standard broad-host-range with CprK1/P_{DB3} expression system	This study
pSEVA247M	Km^R, *oriV* ColE1, *oriT*; standard broad-host-range with msfGFP reporter	Lab collection
pSEVA2311M	Km^R, *oriV* pBBR1; pSEVA2311-derivative with msfGFP cloned as *Hind*III/*Spe*I	Lab collection
pSEVA237R	Km^R, *oriV* pBBR1, *oriT*; standard broad-	Lab collection

	host-range with mCherry reporter	
pSEVA2312R	Km^R, *oriV* pBBR1, pSEVA2312-derivative with mCherry cloned as *Hind*III-*Spe*I	This study
pEM7-crc	Km^R, *oriV* RK2 ,*oriT;* XylR *crc* insensitive variant (see **Table 4**)	Lab collection
pTn*7*-R1a	Gm^R, Km^R, *oriV* R6K, *oriT;* pTn*7*-M-derivative, msfGFP translational fusion to *Pr*-XylR	This study
pTn*7*-R1b	Gm^R, Km^R, *oriV* R6K, *oriT;* pTn*7*-M-derivative, msfGFP translational fusion to *PEM7*-XylR	This study
pTn*7*-R2a	Gm^R, Km^R, *oriV* R6K, *oriT;* pTn*7*-M-derivative, msfGFP translational fusion to *Pr*- XylR mutated in *crc* site	This study
pTn*7*-R2b	Gm^R, Km^R, *oriV* R6K ,*oriT;* pTn*7*-M-derivative, msfGFP translational fusion to *PEM7*-XylR mutated in *crc* site	This study
pYhjH	Tc^R, *oriV* pBBR1-MCS3 with *yhjH* gene of *E. coli* cloned	Gjermansen et al., 2006
pYedQ	Tc^R , pRK404A with *yedQ E. coli* gene cloned	Ausmees et al., 2001
pS-eDQ	Km^R, *oriV* pBBR1, *oritT;* pSEVA2311-derivative with *yedQ* gene cloned as *Avr*II-	This study

pS-eDQ	Km^R, *oriV* pBBR1, *oritT;* pSEVA2311-derivative with *yedQ* gene cloned as *Avr*II-*EcoR*I fragment	This study
pS-hjH	Km^R, *oriV* pBBR1, *oriT;* pSEVA2311-derivative with *yhjH* gene cloned as *Sac*I-*Bam*HI	This study
pSEVA424	Sm^R, *oriV* RK2, *oriT* with $LacI^q$/*Ptrc* expression system	Durante-Rodriguez et al., 2014
pS-lacH	Sm^R, *oriV* RK2, pSEVA424-derivative with *yhjH* gene cloned as *Sac*I-*Bam*HI	This study
pSEVA4413	Sm^R, *oriV* ColE1, *oriT*; with P_{EM7} cloned as *Avr*II/*Pac*I	Lab collection
pAHDO	Sm^R, *oriV* RK2, *oriT,* XylS/*Pm,* with *AHDO* synthetic operon	Nikel and de Lorenzo, 2013a
pSAHDO	Sm^R, *oriV* RK2, *oriT;* pSEVA4413 derivative with *AHDO* operon cloned as *Kpn*I/*Hind*III ,	This study
pSnox	Km^R, *oriV* pBBR1, *oriT*; pSEVA2312 derivative with *nox* gene from *S. pneumoniae* cloned as *Bam*HI/*Pst*I	This study

4. Construction of bacterial strains

Cloning procedures were described by (Sambrook et al., 1989) DNA was amplified with the polymerase chain reaction (PCR) by mixing 50-100 ng of the template

with 50 pmol of each primer and Pfu polymerase (Promega) in a 50 μL reaction volume. The mixture was subjected to 30 cycles of 4 min at 94ºC, 1 min at 55ºC and 1:30 min at 72ºC. Primers were purchased from Sigma-Aldrich. Plasmid DNA was isolated from bacterial cells using the commercial QIAprep Spin Miniprep Kit (Qiagen). PCR-amplified DNA and agarose DNA extractions were usually purified with the NucleoSpin Extract II kit (MN). PCR products or plasmid DNA, restriction endonucleases were purchased from New England Biolabs (NEB). For DNA ligation, T4 ligase (Roche) was used. To transfer recombinant DNA into *E. coli* cells, a chemically competent cell was used with heat shock. For *P. putida*, the manipulated DNA was incorporated by conjugative triparental mating using the *E. coli* HB101 (pRK600) as helper strain. The primers for the PCR reaction in this study are described in **Table 3**. The procedure of each construction was as follows.

4.1. Assembly of bicistronic reporter system

To engineer the dual reporter system, the GFP variant from the pGreenTIR vector (Miller and Lindow, 1997) was PCR-amplified using primers 5-GFP and 3-GFP to generate a 767 bp fragment. This fragment was cloned with *Pst*I/*Hin*dIII into pSEVA236, previously digested with the same enzymes. The resulting vector, termed pGLR1, has a tandem GFP-*luxCDABE* fragment downstream of a multiple cloning site. For the cassette encoding the stable GFP variant, the ~6.6 Kb fragment of pGLR1 spanning the GFP-*luxCDABE* segment was excised with *Sph*I/*Spe*I restriction enzymes and cloned into a pSEVA221, generating vector pGLR2. To validate the dual reporter system, two different promoters were assayed. First, the *Ptrc*-based expression system from pSEVA224 was cloned into pGLR2 as a *Pac*I/*Avr*II fragment, generating the pGLR2-*Ptrc* plasmid. Similarly, a ~500 bp *Pb* promoter from the benzoate degradation pathway of *P. putida* was PCR-amplified using primers 5-PB and 3-PB and cloned as an *Eco*RI/*Bam*HI fragment into pGLR2, to generate the pGLR2-*Pb* plasmid. This plasmid was then mobilized into *P. putida* strain KT2440, which has all native regulatory elements necessary to trigger *Pb* promoter activity in response to benzoate (Cowles et al., 2000). DNA sequencing in all cases confirmed the correctness of cloned fragments.

4.2. Assembly of mini-Tn*7* vectors

To engineer the new miniTn*7* vectors, we designed a DNA segment flanked by restriction sites *AscI/SwaI* and composed of terminal sequences of Tn*7* (*Tn7L* and *Tn7R*) Gm resistance, two terminators T0 and T1, and a multi-cloning site compatible with SEVA architecture (Silva Rocha et al., 2013). This sequence was previously assembled in synthetic pMK (kanamycin-resistant) using *Xho*I and *Asc*I cloning sites. We subsequently digested the Tn*7* fragment as *Asc*I/*Swa*I and ligated it into the *R6K* vector pSEVA211 to obtain pTn*7*-M. To assemble pTn*7*-BG, we edited the translational coupler BCD2 (Mutalik et al., 2013a) to obtain a standardized module compatible with MCS of mini-Tn*7*s. We also included msfGFP as a reporter gene, placed downstream of BCD2 and included between two *Eco*RI sites (**Fig. 6).** The entire fragment BCD2-msf-GFP (BG) was then cloned as *Pac*I/*Sac*I into pTn*7*-M. All promoters and regulation cassettes were cloned as *Pac*I/*Avr*II fragment. *PEM7* was digested from pSEVA2513, *Pm* was amplified from *Pseudomonas putida* mt-2 using primers 5-Pm and 3-Pm, *xylS-Pm* was amplified from pSEVA228 using primers 5-XylS and 3-Pm. Resulting plasmid were named pTn*7*-*PEM7*, pTn*7*-*Pm* and pTn*7*-BG. pTn*7*-SynPro collection was obtained in collaboration with Lara Eisenbach and Sebastian Zobel, (Institute of Applied Microbiology RWTH University of Aachen, Germany); promoters (see **Table 4**) were cloned into pTn*7*-M as *Avr*II/*Pac*I fragments. All cassettes obtained included between Tn*7*L and Tn*7*, were mobilized to *P. putida* KT2440 chromosome by triparental mating as described (Keen et al., 1988). Such insertions occur in the 95% of cases at the *attTn7* site (Choi and Schweizer, 2006) and in the same orientation, thus generating entirely equivalent strains (Bao et al., 1991). To verify correct transposon insertion into the *att* site, some clones resistant only to Gm were selected and PCR-amplified using these combinations of primers: 5-Pput-glmS UP with 3-Tn7L and 5- Ppu-glmS DOWN with 3-Tn7R; the amplification products showed a size of 400 and 200 bp, respectively (Schweizer, 2001). To obtain pTn7-*Pu*GFP, we first cloned *GFP* as a *Hind*III/*Pst*I fragment into pTn7-M; Pu was further digested as *Eco*RI/*Bam*HI from pEZ9 plasmid (de Lorenzo et al., 1991) and

inserted into pTn7-GFP.

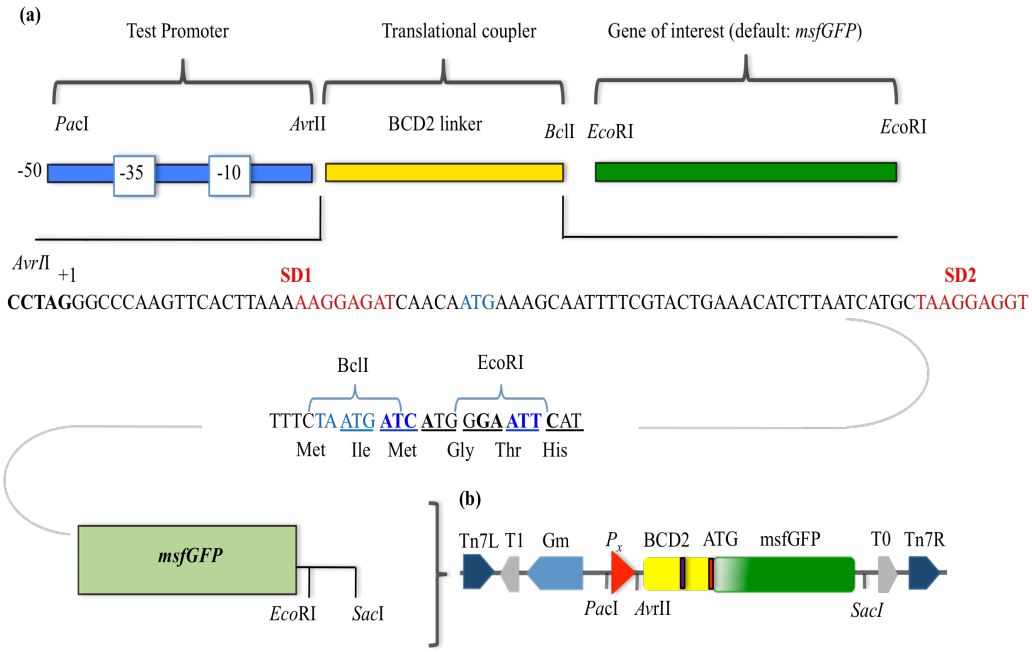

Fig. 6. Structure of standardized module cloned into pTn*7* vectors. A module leading the BCD2 translational coupler (Mutalik et al., 2013a) and msfGFP was designed to be compatible with standardized mini-Tn*7*s. **(a)** The test promoter is always included between *Pac*I/*Avr*II sites; the linker is placed between *Avr*II and *Bcl*I and is coupled with msfGFP. This last is inserted between two *Eco*RI sites, to facilitate its replacement with other reporters or other gene of interest. **(b)** Fragment inserted into mini-Tn7 vector.

4.3. Assembly of translational msfGFP fusions to XylR

Hybrid fragments from msfGFP and *Pr*-XylR were amplified by PCR and used to generate pTn*7*-R1a. 5-Pr and 3-XylRT oligonucleotides were used to obtain the first fragment (F1), using *Pr*-XylR as template; msfGFP served as template to amplify F2 fragment, using 5-msfGFPT and 3-msfGFP. To assemble the final construct, F1 (495 bp) and F2 (710 bp) were used as templates to amplify the translational fusion using 5-Pr and 3-msfGFP as primers, and the entire segment (1.2 Kb) was cloned into pTn*7*-M as *Pac*I/*Bam*HI. pTn*7*-R1b was obtained similarly, substituting *Pr* with *PEM7*, which was previously inserted in the intermediate plasmid pTn*7*-XylR as a *Pac*I/*Avr*II fragment. pTn*7*-*PEM7*-XylR was used as template to obtain the first hybrid fragment F1 and was amplified by

primers 5-PEM7 and 3-XylRT. The second fragment was obtained using msfGFP as template and was amplified by 5-msfGFPT and 3-msfGFP. For the final hybrid construct, F1 and F2 served as templates to amplify the 892 bp fragment with primers 5-PEM7 and 3-msfGFP. A Crc-insensitive variant of XylR was taken from existing plasmid pEM7-crc; in this case, to obtain pTn*7*-R2a, XylR *crc* variant (XylR2) was previously PCR-amplified with 5-XylR and 3-XylR and subcloned into pTn*7*-M as *Eco*RI/*Bam*HI. The *Pr* promoter was then inserted into pTn*7*-R2b as *Pac*I/*Avr*II fragment and the resulting construct, pTn*7*-*Pr*-XylR2, served as a template for obtaining msfGFP translational fusion to XylR2. For pTn7-R2b, fragment pEM7-crc was the template to generate the hybrid with msfGFP and the entire block was then cloned into pTn7-M as *Sac*I/*Bam*HI. For pTn*7*-XylRmT1 and pTn*7*-R2b, DNA assembly strategy was the same as that used to obtain the respective plasmids with the wild type form of XylR.

4.4. Construction of pSEVA with new expression systems

To obtain pSEVA2311, the fragment carrying *chnR* and P*chnB* was designed as standard SEVA cargo (*Pac*I/*Avr*II). The sequence encoding ChnR, including a putative site for promoter binding, was taken from *Acinetobacter* genome (Steigedal and Valla, 2008), and was amplified using oligonucleotides 5-ChnR and 3-ChnR and sub-cloned into pSEVA231 as a *Pac*I/*Avr*II fragment. The promoter sequence leading a linker with the additional promoter P_{Km} (Arce *et al.* in preparation) that constitutively drives *chnR* expression was then inserted as *Nhe*I/*Avr*II into the intermediate plasmid pSEVA231-ChnR. For plasmid validation experiments, msfGFP was excised from pSEVA237M as *Hind*III/*Spe*I and cloned into pSEVA2311, giving rise to pSEVA2311M. A similar procedure was followed to assemble pSEVA2312; the *cprK1* coding sequence was taken from plasmid pMLK1 (Kemp et al., 2013), amplified with primers 5-CPRK1 and 3-CPRK1, and subcloned into pSEVA231. The promoter P_{DB3} was designed with a linker leading the constitutive P_{Km} promoter that controls *cprK1* expression, and this fragment was cloned as *Nhe*I/*Avr*II into pSEVA231-CPRK1. To obtain pSEVA2312R, mCherry was digested from pSEVA237R as *Hind*III/*Spe*I and cloned into

pSEVA2312. To obtain pSnox, the *nox* gene from *Streptococcus pneumoniae* (Auzat et al., 1999) was excised from pSlac-n, purified and cloned as *Pst*I/*Bam*HI fragment into pSEVA2312.

4.5. Construction of plasmids used in catalytic biofilm

To obtain pS-eDQ, the gene for cyclase *yedQ* was amplified by PCR from pYedQ (Ausmees et al., 2001) using 5-YeDQ and 3-YeDQ and cloned into pSEVA2311 as *Avr*II/*Eco*RI fragment. To assemble pS-hjH and pS-lacH, the gene for phosphatase 7*yhjH* was amplified from pYhjH (Gjermansen et al., 2006) as *Sac*I/*Bam*HI and cloned into pSEVA2311 and pSEVA424, respectively. All resulting constructs were confirmed by DNA sequencing.

5. Bioluminescence and fluorescence assays of whole populations

Emission of fluorescence and light in cells growing on Petri dishes were recorded with a luminometer VersaDoc imaging system Model 4000 (BioRad). Images were captured and processed with Quantity One 4.6.9 analysis software (BioRad). For quantitative promoter activity assays, single colonies of *E. coli* and *P. putida* reporter strains were picked from fresh plates and inoculated into 5 mL of medium with appropriate antibiotics. Cells were then grown overnight at 170 rpm. After pre-growth, the cells were washed twice with 10 mM $MgSO_4$ buffer and diluted to an optical density of OD_{600} of 0.1 into fresh medium and, when required, with different inducer concentrations. Microtest 96-well assay plates (BD Falcon) were filled with 200 µL per well of diluted culture and placed in a Wallac Victor 2 Microplate Reader (Perkin Elmer). Plates were then incubated with shaking for several hours. At time intervals of 30 min, the $_{OD600}$ and the luminescence or the fluorescence of each culture was measured. Non-inoculated medium was used as a blank for adjusting the baseline for measurements. Promoter activity was calculated by normalizing reporter signals (luminescence or fluorescence) to the OD600 readings. Data were processed using Microsoft Excel 2010 and MATLAB software (MathWorks).

6. Single-cell analysis by flow cytometry

Single-cell experiments were performed with flow cytometers. For GFP analysis, a Gallios flow cytometer (Perkin Elmer) was used; GFP was excited at 488 nm and the fluorescence signal recovered with a 525(40) BP filter. The MACSQuant VYB (Miltenyi) flow cytometer was used to analyze mCherry signal in pSEVA2312R; mCherry was excited at 561 nm and the emission signal was recovered with a 615/20 BP filter. For induction experiments, cells grown overnight were diluted 1/50 in fresh M9 media containing the appropriate carbon source and incubated for 4-5 h. After this pre-incubation, at the mid-exponential phase, the cells were divided into two samples; one was induced by the corresponding compound and the other was used as a non-induced control. Cultures were then incubated with shaking in air at the appropriate temperature, and each hour after induction, an aliquot of each sample was stored on ice until analysis. For analysis of GFP in translational msfGFP fusions to XylR, *P. putida* KT2440 cells were grown overnight at 30ºC and diluted 1/100 in LB medium containing Gm as antibiotic. Cells were incubated at 30ºC with shaking air and at early exponential phase, an aliquot of samples was analyzed every 15 min until the OD_{600} reached the value of 1.0. For each aliquot, a maximum of 30,000 events were analyzed. The data were processed using FlowJo v.9.6.2 software.

7. Sample preparation for microscopy

For single cell analysis, *P. putida* KT2440 cells were grown overnight in 20 mM M9 succinate at 30ºC and then diluted 1/50 in the same medium with the appropriate inducer. At mid-exponential phase (OD_{600} 0.4), cells were exposed to *m*-xylene vapors and incubated at 30ºC. Every hour thereafter, a sample was taken and stored on ice. After 3 h, cells were prepared for microscopy; the samples were placed on poly-L-lysine (Sigma)-coated coverslips to immobilize the reporter cells for observation. The coverslip was sealed with nail polish in addition of ProLong (Invitrogen) to inhibit fluorescence bleaching (Kim et al., 2013). For biofilm analysis, a glass coverslip (22 mm x 22 mm; Mentel-Glaser) was placed in a tilted

position in a six-well plate (Thermo Scientific) with 5 mL M9 medium supplemented with glucose, antibiotics and inducer, inoculated with *P. putida* KT2440 Δall-θ with OD_{600} of 0.05 (Xavier et al., 2009). After growth at room temperature without agitation, coverslips were recovered and washed 3 times with H_2O, then sealed as above. Coverslips were washed with SDS detergent solution to obtain a one-dimensional image of the biofilm surface. All images were acquired on an epifluorescence microscope (Olympus BX61) with a cooled CCD camera and processed with ImageJ software.

8. Biofilm quantification

Biofilm formation of *P. putida* KT2440 was quantified by the crystal violet assay as described (O'Toole et al., 2000; Tolker-Nielsen et al., 2000). Cells were grown in multi-well plates with M9 medium supplemented with required antibiotics, carbon sources and different inducer concentrations, and allowed to grow 24 h at 30ºC without shaking. The OD_{600} of suspended cells was then measured by SpectraMax (BioNova Scientific) and all culture liquid was removed. After washing wells three times with H_2O, a 0.1% crystal violet solution was added to the plates, which were incubated 30 min at room temperature. After discarding the crystal violet, the remaining solution associated with biofilms was dissolved by addition of 15 mL of a 33% (v/v) acetic acid solution. Plates were agitated gently for 1 h. Crystal violet density was acquired in SpectraMax at a wavelength of 590 nm, and these values were normalized to the initial OD_{600} of each culture. Data were analyzed using Microsoft Excel 2010.

9. Dehalogenase assay

Haloalkane dehalogenase assays were performed by incubating *P. putida* KT2440 cells in multi-plate wells with M9C (M9 medium with low chloride content), added with glucose or succinate as the carbon source, and the appropriate inducer for 48 h at room temperature without shaking. Halide liberation was monitored colorimetrically with the method of Bergmann and Sanik (1957), which depends on displacement of the thiocyanate ion from mercuric thiocyanate by inorganic

chloride (Nikel and de Lorenzo, 2013a). For routine determinations, 1-chlorobutane was used as the substrate. Halide production (in the 0 to 1 mM range) was determined spectrophotometrically at 460 nm. Dehalogenase activity in recombinants was screened by incubating the strains to be tested in a microtiter plate with 100 μL of a mixture of 5 mM 1-chlorobutane in 50 mM Tris·H_2SO_4 buffer (pH 8.2). After overnight incubation of the plate at room temperature without agitation, 100 μL 0.25 M $NH_4Fe(SO_4)_2$ in 6 M HNO_3 was added, followed by a drop of saturated $Hg(SCN)_2$ in ethanol.

IV. RESULTS

Chapter I

Design of standardized genetic tools for gene expression analysis in Gram-negative bacteria

The content of this section has been published as:

Benedetti I., de Lorenzo V. and Silva-Rocha R., Quantitative, non-disruptive monitoring of transcription in single cells with a broad-host-range GFP-luxCDABE dual reporter system, *PloS One* 7(12), 2012.

Benedetti I. and de Lorenzo V. Promoter fusions with optical outputs in individual cells and in populations, *Methods in Molecular Biology*, vol. 1149, chapter 4, 2014.

Part of this section is in preparation as:

Benedetti I. and de Lorenzo V. Analysis of promoter activity in *P. putida* using a standardized set of mini-Tn*7* vectors.

Benedetti I., Nikel P.I., de Lorenzo V. A standardized CprK1/P_{DB3} expression device to tightly regulate redox homeostasis.

Background

Standardization of biological parts is one of the most frequently discussed issues in the scientific community, given the importance of engineering many components of synthetic systems (Ellis et al., 2011). Different groups have organized diverse platforms that allow grouping of a series of standardized genetic tools. A typical example is the Biobrick Foundation, where synthetic biological parts such as promoters, terminators, RBS or entire devices are registered and made available for public use (Smolke, 2009). Another more recent example is the pSEVA platform, an extended database (SEVA-DB (Silva-Rocha et al., 2013), of standards for vector assembly (Durante-Rodriguez et al., 2014). pSEVAs are organized with a modular architecture, in which each part is a genetic component such as antibiotic markers, two terminators, origin of replications, one origin of transfer and variable cargo. These six functional modules are inserted between two unique restriction sites, which permit their interchange or reuse. Based on this concept, we designed several genetic tools that included plasmids and transposons, which we optimized for Gram-negative bacteria. First, we developed a number of plasmids bearing the transcriptional fusion to the bicistronic reporter system GFP-*luxCDABE*, which facilitates promoter analysis in populations and in single cells, (Benedetti I.M., 2012) . We next engineered standardized mini-Tn*7* vectors, and focused on developing a reliable monocopy system. Finally, we tested new expression systems such as SEVA cargos, and tested which mechanism responds to unusual compounds such as chlorinated chemicals.

1. Quantitative, non-disruptive monitoring of transcription in single cells with a broad-host range GFP-*luxCDABE* dual reporter system

The work presented here shows the implementation and validation of a novel GFP-*luxCDABE* dual reporter system suitable for promoter probing in Gram-negative bacteria (Benedetti and de Lorenzo, 2014; Benedetti I.M., 2012). As this system is based on broad host-range vectors, it can be used in a wide range of microorganisms. Additionally, the advantages of combining the GFP and *lux*

reporters in a single cassette makes this dual system a powerful tool for the study of gene regulatory networks in native conditions using a single cloning step.

To generate a new dual reporter system, we cloned the GFP *tir* variant (Miller and Lindow, 1997; Ropp et al., 1995) as an *Sph*I/*Hind*III fragment into the broad host-range plasmids pSEVA236 and pSEVA226 (Table 2), which bear the complete *lux* operon from *Photorhabdus luminescens* (*luxCDABE*) (Winson et al., 1998)and see Material and Methods). The resulting vectors, termed pGLR1 and pGLR2 (**Fig. 7a,** see **Table 2**), harbor the Km resistance marker and either a *pBBR1*- (for pGLR1) or *RK2*-based origin of replication (for pGLR2) (Bagdasarian et al., 1981; Keen et al., 1988; Thomas et al., 1984), both of which replicate in a large number of hosts. Both reporter systems use the same optimized ribosome-binding site (the *tir* element (Miller and Lindow, 1997) preceding the start codon. In the case of *lux*, the *tir* element is placed upstream of the first gene of the operon (*luxC*). The dual cassette is located downstream of an extensive multiple cloning site (MCS) composed of 12 unique restriction sites used for promoter cloning (**Fig. 7b**). An identical GFP-*lux* dual construct vector bearing an unstable GFP protein (lva, (Andersen et al., 1998) was engineered for transient expression studies. To evaluate the performance of the GFP-*luxCDABE* cassette in assessing promoter induction kinetics, we analyzed expression of the *Ptrc*- and *Pb*-based constructs in response to their cognate inducers in *E. coli* and *P. putida*, regulation mechanisms in bacteria. In the first system, the LacI protein represses *Ptrc* activity by blocking binding of the RNA polymerase (RNAP) to this promoter Amann et al., 1988) (Aksoy et al., 1984). This promoter is expressed when the inducer (e.g., lactose, IPTG) binds to LacI and removes it from *Ptrc*, allowing transcription (Amann et al., 1988). In the case of *Pb*, the transcriptional activator BenR triggers promoter activity when bound to the inducer benzoate (Cowles et al., 2000). By fusing these two systems to the GFP-*luxCDABE* reporter, we assayed both transcriptional repression and activation in the two model organisms.

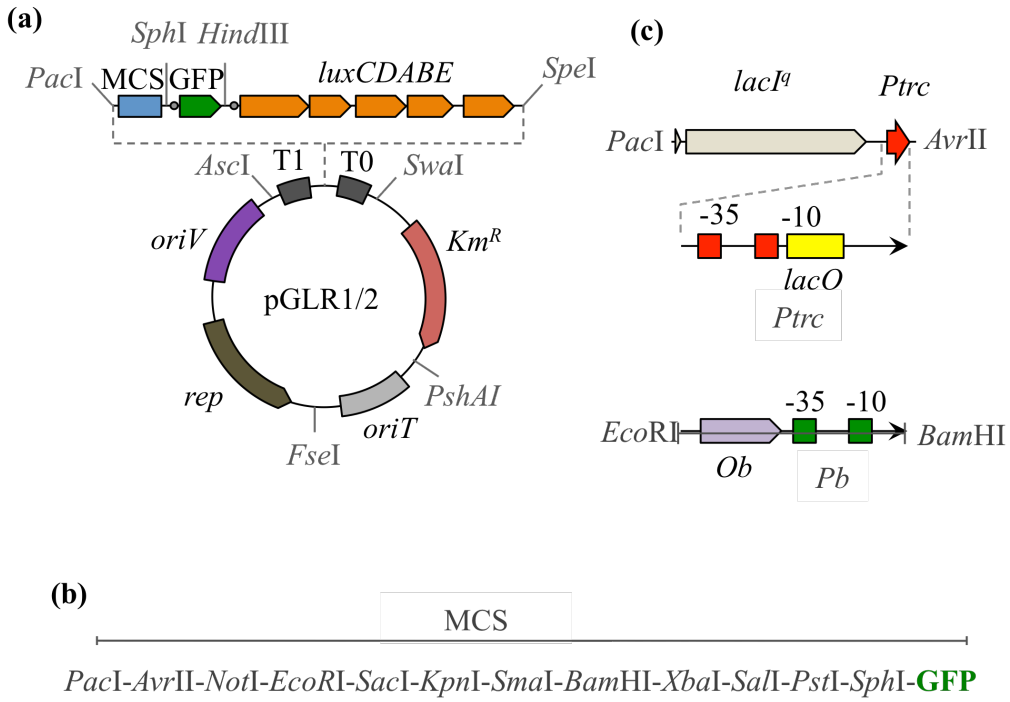

Fig 7. Structural organization of pGLR1/2 vectors and expression systems assayed. (a) Each vector harbors a kanamycin resistance (Km^R) marker, an *oriT* for plasmid transfer through conjugation, and a broad host-range origin of replication that consists of a vegetative origin (*oriV*) and a replication protein (*rep*). Vector pGLR1 is based on a minimal pBBR1 origin (Twigg and Sherratt, 1980), whereas pGLR2 is based on ori RK2 (Lale et al., 2011; Thomas et al., 1984). The GFP-*luxCDABE* reporter cassette is cloned between two strong terminators (T0 and T1) and is downstream of a MCS. The optimal ribosome-binding site of the *tir* element (grey circle (Miller and Lindow, 1997) is placed upstream of the *gfp* gene and the first gene of the *lux* operon (*luxC*). **(b)** List of enzymes found at the MCS. **(c)** Architecture of the $LacI^q$/*Ptrc* expression system (top) and the *Pb* promoter (bottom). Relevant features such as operators (*lacO* and *Ob*) and promoter (-10/-35) regions are represented.

For these tests, cells grown overnight were diluted in fresh medium containing increasing concentrations of the specific inducer (IPTG or benzoate) and assayed in a multi-label plate reader (see Material and Methods). At 30-min intervals, the OD_{600} and luminescence were recorded. **Figures 8a** and **8b** show the induction profiles of the two expression systems in response to increasing concentrations of the inducers.

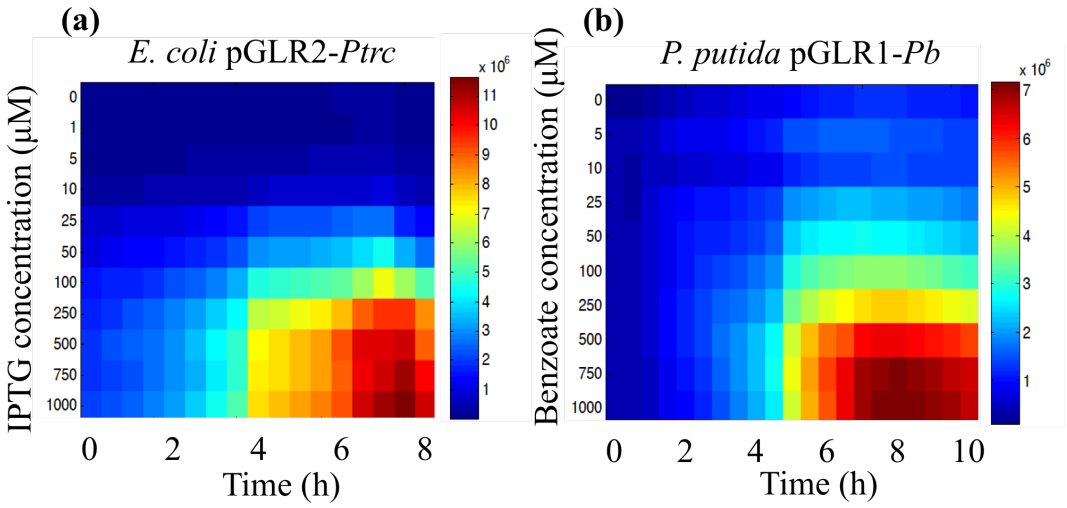

Fig. 8. Expression landscape using a bioluminescence signal in *E. coli* and *P. putida* hosts. Strains grown overnight were diluted 1:20 in fresh medium containing different concentrations of IPTG or benzoate, as indicated. At 30-min intervals, bioluminescence and OD_{600} signals were recorded. Figures show the level of promoter activity for each strain over time. *E. coli* CC118 harboring pGLR1-*Ptrc* (see **Table 2**) **(a)** was induced with IPTG, while *P. putida* KT2440 with pGLR2-*Pb* (see **Table 2**) was induced with benzoate **(b)**. Color bars (left) indicate the scale for promoter activity.

To validate the potential of the dual GFP-*luxCDABE* reporter cassette as a tool for assessing the single-cell behavior of target promoters, we examined the expression profiles of the *Ptrc*- and *Pb*-based systems in response to IPTG or benzoate, respectively, by analyzing the GFP reporter. For this analysis, cells grown overnight (*E. coli* with pGLR2-*Ptrc* and *P. putida* with pGLR1-*Pb*) were diluted in fresh medium and allowed to grow to mid-exponential phase. Each reporter strain was then exposed to its specific inducer and incubated for several hours. At 1-h intervals, samples were taken and analyzed by flow cytometry (see Material and Methods). Both expression systems showed unimodal behavior at the single-cell level (**Fig. 9a, 9c**). These results coincide with previous reports on the behavior of *Ptrc* and *Pb* systems, and show that the synthetic vectors used to implement the dual reporter cassette do not interfere with the native activities of the target promoters. On the basis of these results, we conclude that this dual system could be useful for promoter targeting in populations and in single cell.

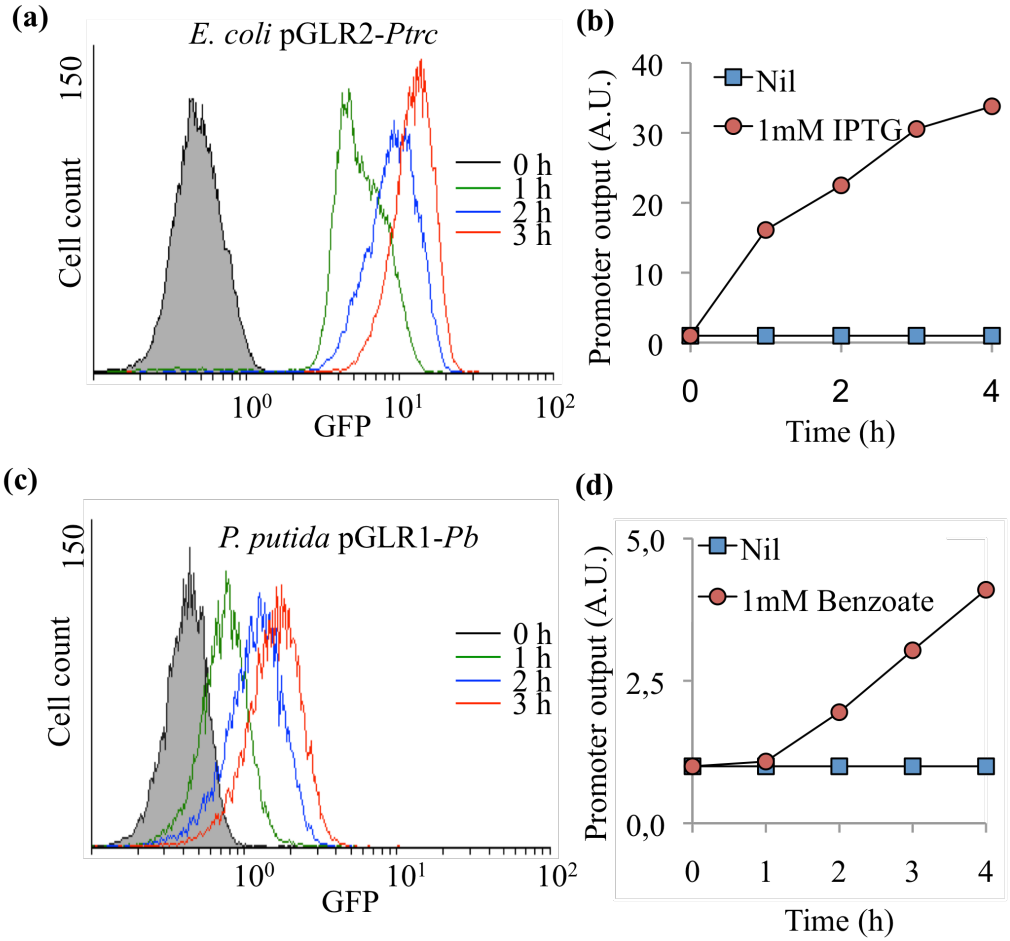

Fig. 9. Single-cell analysis of *Ptrc*- and *Pb*-based systems. Strains grown overnight were diluted 1:20 in fresh medium and allowed to grow to mid-exponential phase. At this point, IPTG at 1.0 mM for *E. coli* harboring the *Ptrc::*GFP-*luxCDABE* system **(a)** or benzoate at 1.0 mM for *P. putida* with *Pb::*GFP-*luxCDABE* **(b)** were added to the culture. At 1-h intervals, samples were collected and stored on ice until analysis by flow cytometry. Untreated cells were used as controls. For each assay, 15,000 cells were analyzed. **(c)** Induction profile of *E. coli Ptrc::*GFP-*luxCDABE* strain in response to IPTG at 1.0 mM. **(d)** Induction profile of *P. putida Pb::*GFP-*luxCDABE* strain in response to benzoate at 1.0 mM. Profiles in **(c)** and **(d)** were calculated by normalizing mean fluorescence levels of induced populations to fluorescence levels of the untreated control samples.

2. Analysis of promoter activity in *Pseudomonas putida* using a standardized set of mini-Tn*7* vectors

In another approach, we developed a series of standardized vectors whose function is based on the Tn*7* mechanism. Tn*7* is a transposon able to insert specifically into the chromosome of several bacteria (Bao et al., 1991). In 95% of cases, the transposition occurs in a region located downstream of gene *glmS*, conserved in

many Gram-negative bacteria, that codes for the enzyme glucosamine-fructose-6-phosphate aminotransferase (Peters and Craig, 2001). Transposition takes place when the transposase complex recognizes and cleaves Tn*7* 3' and 5' ends, and mediates insertion into the Tn*7*-specific *att* site (Choi and Schweizer, 2006; Choi et al., 2013; Craig, 2001; Peters and Craig, 2001). As a result of this specific and in most cases neutral chromosomal insertion, mini-Tn*7*-based vectors have proven useful for various genetic applications including gene expression analysis (Choi et al., 2005), functional characterization of genes (Damron et al., 2013), and single copy gene complementation (Schweizer, 2001). The scarcity of these types of standardized tools makes their application difficult; we therefore designed standardized and minimized Tn*7* vectors, which reflect SEVA architecture. As a backbone vector, we used a *R6K* suicide pSEVA211 (Stalker et al., 1982) and cloned a mini-Tn*7* cargo at the *Asc*I/*Swa*I site (**Fig. 10a;** see **Table 2**); this module has two Tn*7* extremes, two terminators T1 and T0, a Gm marker and the SEVA variant multi-cloning site (**Fig 10b**; (Silva-Rocha et al., 2013). Because the principal purpose of this work was to obtain a monocopy system optimized for the Gram-negative microorganism *P. putida* KT2440, we implemented an additional part that consists of a translational coupler and a reporter gene, in this case msfGFP (Lab collection). We added this segment was to improve GFP production and thus receive a better readout signal from the promoter of interest. To assemble the translational coupler, from an open registry of parts we chose the so-called BCD2 (bi-cistronic design) to improve translation of the gene of interest (GOI), to which it is coupled (Mutalik et al., 2013a). The fragment has two ribosome-binding sites (RBS); the first sequence is constant whereas the second is variable and coupled to the GOI. The function of two Shine-Dalgarno sequences limits interaction of mRNA secondary structures across 5' untranslated regions; this influences binding to ribosomes and thus, translation efficiency (Mutalik et al., 2013b). We selected the unit BCD2 (Kosuri et al., 2013; Mutalik et al., 2013a) and edited it to obtain a standardized fragment compatible with our Tn*7* format (see sequence in **Fig. 6** and **Table 4**).

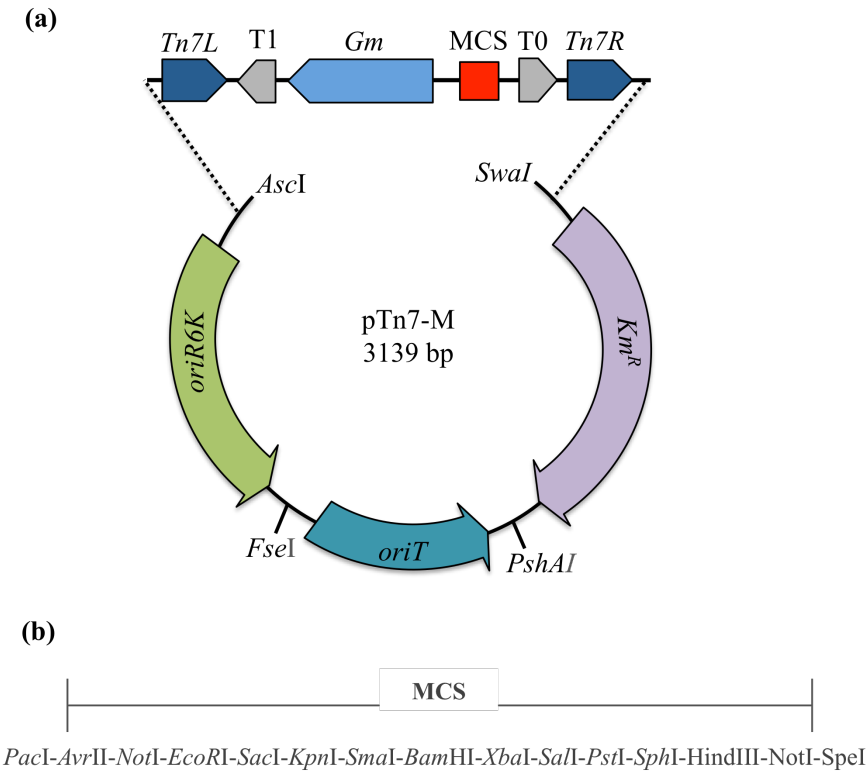

Fig. 10. Structural organization of mini-Tn7 vectors. (a) Functional elements of the plasmid include [i] a backbone vector bearing a Km^R marker, origin of replication *R6K* (Stalker et al., 1982), and the origin of transfer *oriT* and [ii] a Tn*7* module cloned between *Asc*I and *Swa*I sites that bears a Gm^R marker, two terminators T1 and T0, a SEVA version of MCS, and two Tn*7* sites recognized by transposase. **(b)** List of enzymes found at the MCS .

The resulting BCD2-G segment contains an *Avr*II/*Pac*I site for cloning a promoter or an expression system, two RBS (BCD2 linker) included as *Avr*II/*Bcl*I sites, and a translationally coupled msfGFP placed as an *Eco*RI/*Eco*RI fragment (see **Fig. 6** in Material and Methods). To assess the performance of this new construct, we analyzed different promoters in single *P. putida* cells. We first evaluated the effect of the BCD2 linker for GFP production, for which we compared the activity of synthetic and constitutive *PEM7* (Lane et al., 2007), alone or with BCD2. After transposon insertion (see Material and Methods), cells of *P. putida* KT2440 were grown in M9 citrate medium overnight at 30ºC. The following day, strains were diluted in 1X PBS to an OD_{600} of 0.3 and analyzed by flow cytometry. GFP

activity was improved by 20-fold when the translational coupler was present **(Fig. 11a, 11b)**.

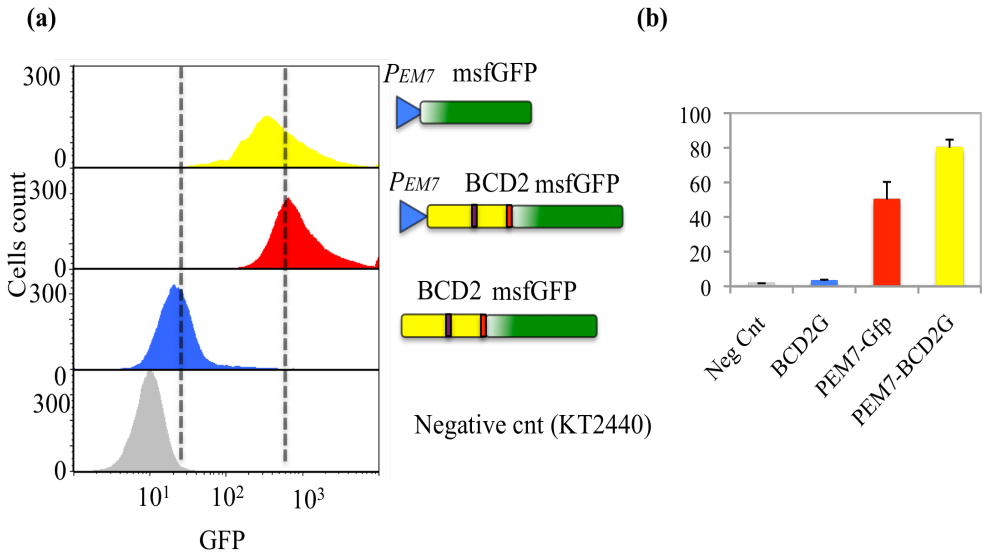

Fig. 11. GFP quantification in Tn*7*-BG monocopy systems. **(a)** Modules bearing promoter *PEM7*, translational coupler BCD2 and the gene coding for msfGFP were inserted into the *P. putida* KT2440 genome by the Tn*7* mechanism. Cells were grown overnight in M9 medium and then diluted to an OD_{600} of 0.4 in 1X PBS. The efficiency of the BCD2 translational coupler in *P. putida* cells was tested by quantifying the GFP signal by flow cytometry. Promoter *PEM7* drives expression of msfGFP, whose translation is coupled to BCD2. **(b)** Promoter intensity was normalized to mean GFP fluorescence of *P. putida*; we used *P. putida* KT2440 as negative control, and considered a *P. putida* KT2440 with the insertion *PEM7*-msfGFP as the positive control. Bars show the mean values of each parameter ± standard deviation of triplicate measurements in at least two independent experiments.

We used a similar approach to test the activity of the XylS/*Pm* expression system (Kessler et al., 1994a) in single *P. putida* cells and in other contexts. This system is present in the TOL plasmid of the wild type strain of *P. putida* (mt-2), and belongs to the TOL pathway involved in degradation of aromatic compounds, (Gallegos et al., 1997; Marques and Ramos, 1993; Worsey and Williams, 1975; Yano et al., 2010). XylS is the regulator transcribed by its promoter *Ps* and triggers *Pm* activation when bound to its inducer benzoate or to 3-methyl benzoate (3MBz) (Ramos et al., 1997). XylS/*Pm* is widely used in *P. putida* and other bacteria as a heterologous expression system due to its sensitivity to inducers and the intense

activity of *Pm* promoter (Kessler et al., 1994b). In the first case, we inserted *Ps*-XylS/*Pm* into the chromosome of *P. putida* KT2440 (the variant without the TOL plasmid (Nelson et al., 2002) and quantified *Pm* activity through the GFP signal. In the second case, we analyzed *Pm* in *P. putida* mt-2 ((Worsey and Williams, 1975); see **Table 1**), which contains the TOL plasmid and all regulatory components, and *Pm* activity was quantified by GFP signal. In both conditions, *Pm* was induced by 3MBz at various concentrations; in the first case, *Ps*-XylS is placed into the chromosome together with *Pm*, while in the second experiment, the native transcription factor and its promoter are located in the TOL plasmid and activates the additional *Pm* inserted into the chromosome (**Fig. 12a, Fig. 12b**). Cells grown overnight were diluted 1/50 in M9 medium with citrate as a carbon source and 3MBz; at 4 h after addition of the inducer, a small fraction of cells was analyzed by flow cytometry. Distribution of single cells differed in the two cases; in *P. putida* KT2440, we observed homogeneous distribution of induced cells, whereas curve shape for mt-2 indicated greater heterogeneity of *Pm*-expressing strains (**Fig. 13a, Fig. 13c)**. The promoter output also differed, as *P. putida* mt-2 strain showed lower and noisier *Pm* activity (**Fig. 13b, Fig. 13d**), possibly due to the presence of the TOL plasmid (and native *Pm*; (Silva-Rocha and de Lorenzo, 2012b).

As this system appeared to be functional to calibrate expression units, we tested various synthetic promoters engineered for *P. putida* KT2440. We designed and subcloned several synthetic promoters into mini-Tn*7* delivery vectors, inserted them into the *P. putida* KT2440 chromosome (in collaboration with Lara Eisenbach and Sebastian Zobel, Institute of Applied Microbiology RWTH University of Aachen, Germany) and generated the pTn*7*-SynPro collection (see **Tables 1-2**). The experimental procedure was as detailed above; in this case, we used flow cytometry to analyze cells in exponential phase (OD_{600} = 0.4). Most promoters tested showed higher activity than P_{EM7} (reference control), and only one appeared to behave similarly in both growth phases (SynPro34; **Fig. 14**). To conclude this

section, we engineered standardized mini-Tn*7* vectors to generate a reliable monocopy system for gene expression analysis in *P. putida.*

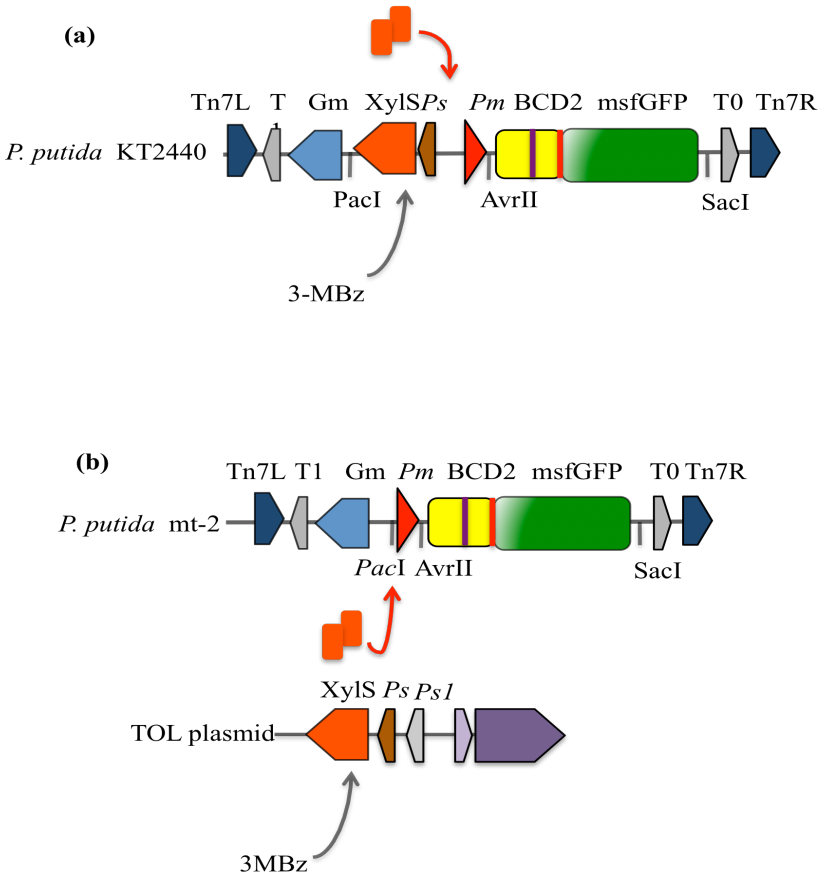

Fig. 12. Mechanisms of *Pm* induction in two *P. putida* strains. *Pm* responds to XylS, which is transcribed by its promoter *Ps* and is activated by 3MBz. **(a)** The *Ps*- XylS/*Pm* cassette was cloned in the mini-Tn*7* vector (pTn7-BG) and all fragments were mobilized to the *P. putida* KT2440 chromosome. **(b)** The *Pm* promoter was cloned into the mini-Tn*7* vector and the fragment transposed to the *P. putida* mt-2 chromosome. This strain bears the TOL plasmid, which encodes genetic elements for degradation of aromatic compounds. In this case, *Pm* responded to native XylS, which was on the TOL plasmid.

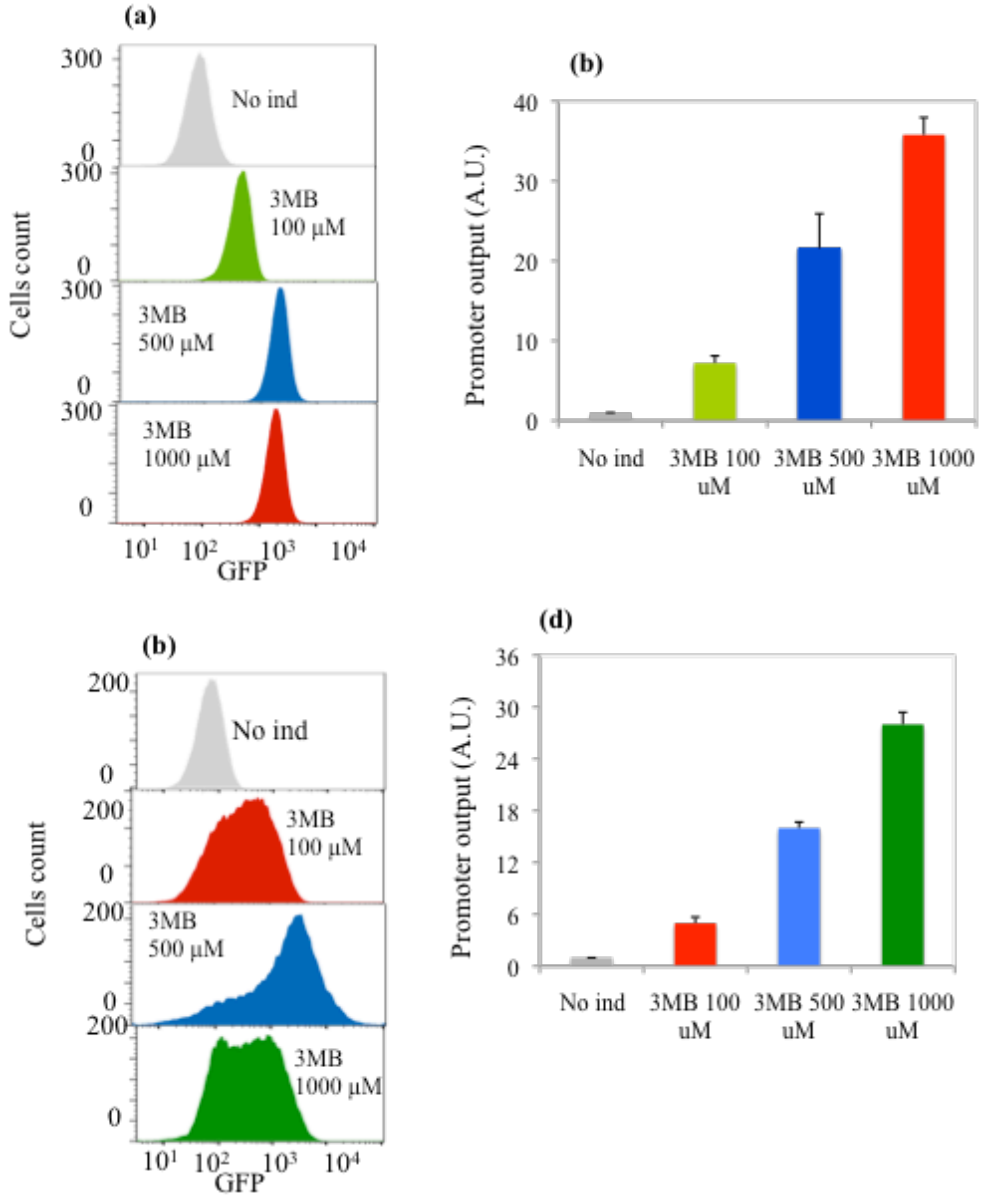

Fig. 13. Analysis of *Pm* activity in single cells of *P. putida*. Strains grown overnight were diluted 1/50 and allowed to grow in M9 citrate medium supplemented with 3MBz at different concentrations. After 4 h, when cells reached mid-exponential phase, samples were collected and analyzed by flow cytometry. For each assay, 20,000 cells were analyzed. Panels **(a)** and **(b)** show the *Pm* induction profile in *P. putida* KT2440 strain, while panels **(c)** and **(d)** show *Pm* activity in the wild type *P. putida* mt-2 strain. Profiles in **(b)** and **(d)** were calculated by normalizing mean fluorescence levels of induced populations to those of untreated control samples. Bars show mean values of each parameter ± standard deviation of triplicate measurements from at least two independent experiments.

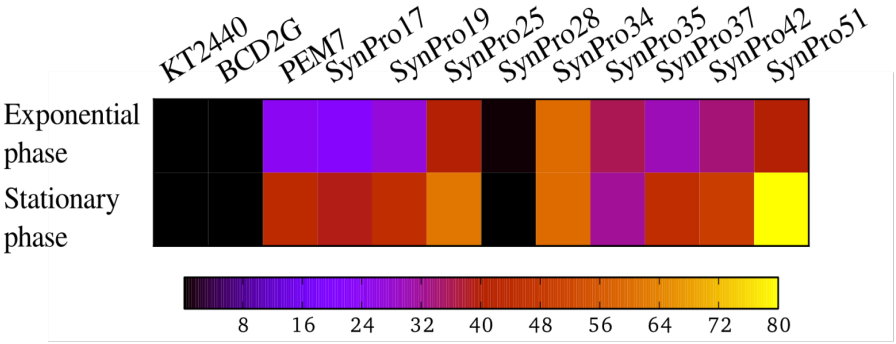

Fig. 14. Calibration of synthetic promoters inserted into the *P. putida* KT2440 genome. Cells bearing chromosomal insertions of synthetic promoters (Tn*7*-SynPro), were grown overnight in M9 citrate medium and then diluted 1/50 in the same medium. Samples were collected at mid-exponential phase and stationary phase (OD_{600} of 1.0) and analyzed by flow cytometry. Cells with no inserted promoter were used as negative controls. Promoter sequences are described in **Table 4.**

3. Design and validation of an expression system compatible with the SEVA format

3.1 Standardizing the CprK1/*PDB3* expression system as a device for precise regulation of metabolic enzymes

CprK is a transcription regulator of the metabolically versatile bacterium *Desulfitobacterium hafniense*, which belongs to the Fnr/Crp family of transcription regulators (Gabor et al., 2006; Kemp et al., 2013). This anaerobic microorganism is able to carry out a process known as reductive dechlorination, in which several types of chlorinated compounds are used as the terminal electron acceptors (Haggblom et al., 2000). As it is active in anoxic conditions, the CprK regulator is inactivated in the presence of O_2. For this reason, we used a variant of this transcription regulator termed CprK1, which bears a mutation in the cysteine residue at position 200 (C200S; (Kemp et al., 2013). This mutation renders CprK1 insensitive to oxidation, and the regulator is activated by exposure to chlorinated intermediates such as 3-chloro-4-hydroxyphenylacetic acid (CHPA;(van de Pas et al., 1999). Once activated, CprK binds to a specific sequence upstream of the genes it controls (the *cprA* gene cluster, which encodes a reductive dehalogenase), termed *dehalobox* (Smidt et al., 2000). This region spans 14-bp inverted repeats and three

variants have been described to date, DB1, DB2 and DB3 (Gabor et al., 2006); we chose DB3 because of its optimal binding site for the CprK1 regulator (Kemp et al., 2013).

We designed a *Pac*I/*Avr*II module that comprises [i] the positive regulator CprK1, [ii] the constitutive P_{km} promoter that controls the expression of the gene that encodes the regulator, [iii] a spacer sequence to insulate regulator expression from the rest of the gene (Durante-Rodriguez et al., 2014) see **Table 4**) and [iv] a promoter recognized by the activated CprK1 regulator (P_{DB3}). All these functional elements were cloned into the broad host-range plasmid pSEVA231 as the backbone vector, to generate pSEVA2312 (see **Table 2**). In addition, we inserted a reporter gene; in this case, we cloned mCherry (Shaner et al., 2004) as a *Hind*III/*Spe*I fragment into pSEVA2312, to obtain pSEVA2312-R (**Fig. 15a;** see **Table 2**). We quantified mCherry by flow cytometry, to assess activity of the P_{DB3} promoter in *E. coli* cells. Strains were grown overnight in different culture media at 37ºC (see Material and Methods); cultures were then diluted 1/50 in appropriate fresh medium. When they reached exponential phase, we added the CHPA inducer at 0.1 mM; samples were taken hourly for the first 4 h and again at 24 h. mCherry formation was quantified by flow cytometry. mCherry maturation time is longer than that of GFP, and we observed maximum output signal after 24 h **(Fig. 15b, 15c)**,(Muller-Taubenberger and Anderson, 2007) . This expression system shows a low basal level (compared with $LacI^q$/*Ptrc*); it is also sensitive to very low inducer concentrations (in the micromolar concentration range; not shown).

3.2 Use of the CprK1/P_{DB3} expression system to manipulate the redox state of cells

As discussed in Section 3.1, we first tested the function of the CprK1/P_{DB3} expression system in *E. coli* using a fluorescent reporter assay. We also used this system to manipulate and monitor the physiological state of *P. putida* and *E. coli* through the controlled expression of *nox* (Vemuri et al., 2006). This gene, from the Gram-positive bacterium *Streptococcus pneumoniae*, encodes a water-forming

NADH oxidase, which decouples the classical pathway for NADH oxidation (oxygen reduction in the respiratory chain) from energy generation (Auzat et al., 1999; Ebert et al., 2011); specifically, it converts O_2 to H_2O with minor formation of H_2O_2 (**Fig. 16**).

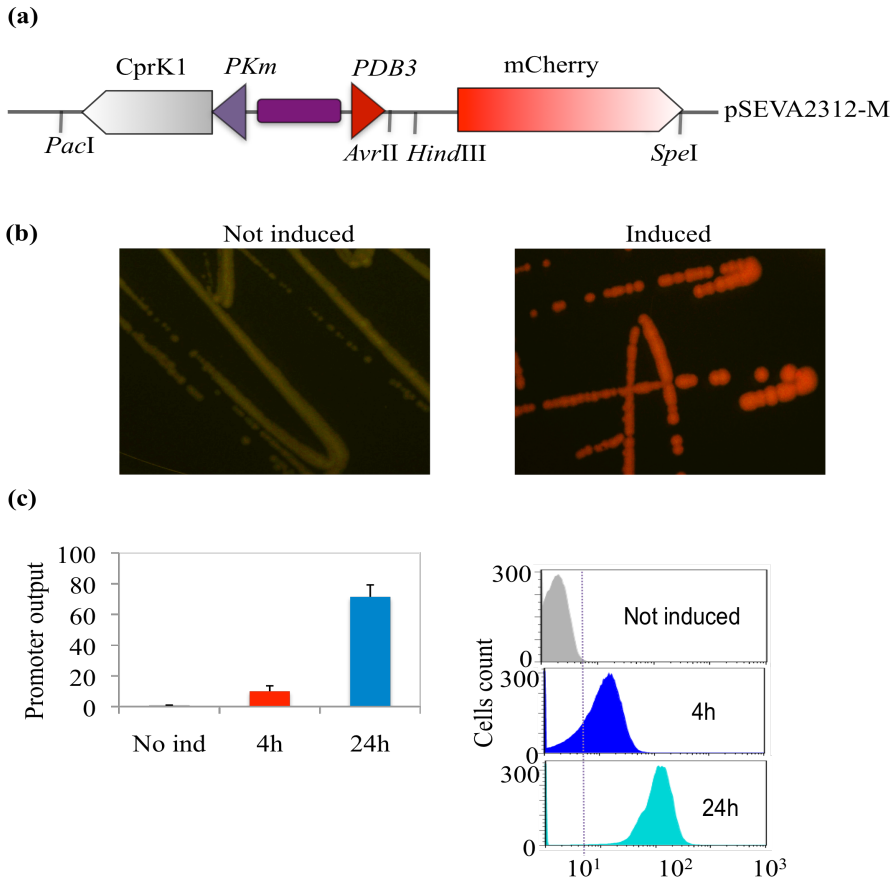

Fig. 15. Structural organization and validation of pSEVA2312-R. **(a)** Functional elements of pSEVA2312-R include [i] the positive regulator CprK1, which responds to the inducer, [ii] promoter *Pkm*, which controls CprK1 expression, [iii] a spacer sequence to isolate regulator expression from the rest of the gene, iv) the promoter *PDB3*, which responds to activated CprK1, and [v] the mCherry reporter gene. **(b)** *E. coli* strains bearing pSEVA2311-R were grown in solid LB medium supplemented with CHPA at 0.5 mM, the CprK1 inducer. After 24 h, we detected mCherry signal and acquired images with a CCD camera. Untreated cells were used as negative control. **(c)** *E. coli* strains carrying pSEVA2312-R were grown overnight on glucose minimal medium; cultures were then diluted 1/50 and allowed to grow to mid-exponential phase. We then added CHPA inducer at 0.1 mM; samples were collected every hour and analyzed by flow cytometry. Induction profiles in **(c)** were calculated by normalizing mean fluorescence levels of induced populations to those of untreated control samples. For each assay, 20,000 cells were analyzed. Bars show mean ± standard deviation for each parameter of triplicate measurements from at least four independent experiments.

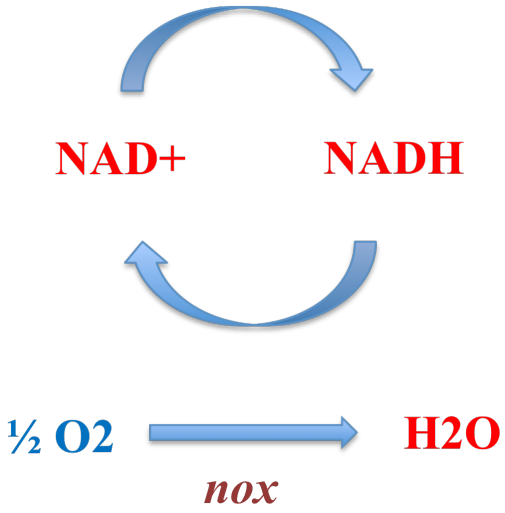

Fig. 16. Reaction catalyzed by NADH oxidase (Nox). Nox is a water-forming NADH oxidase produced in *S. pneumoniae* (Auzat et al., 1999). This enzyme decouples NADH oxidation from respiratory energy generation. Relative to other Nox, this is NADH-specific and converts O_2 to H_2O with minor formation of H_2O_2 (Ebert et al., 2011),(Vemuri et al., 2006) .

Cellular energy demands should thus be unaffected by this enzyme activity, allowing for the specific study of the impact of NADH oxidation on cell metabolism (Rocha-Martin et al., 2011). Since NADH recycling through Nox alters the overall redox homeostasis of cells (specifically, the NADH/NAD^+ ratio), precise regulation of enzyme activity is crucial for preventing toxic effects. To use engineered pSEVA2312 to quantitatively express *nox* in *P. putida* cells, we cloned *nox* from pSlac-n as *Bam*HI/*Pst*I into the pSEVA2312 plasmid and obtained pSnox (**Fig. 17a,** see **Table 2**). In addition, we placed the *nox* gene under the control of $LacI^q$/*Ptrc* (pSlac-n plasmid, see **Table 2**) and compared regulation of the two expression systems. We transformed *P. putida* KT2440 with pSnox, pSlac-n or pSEVA2312 (used as a control) and calculated the specific growth rate as a rough indication of Nox impact (as NADH recycling) on overall cell physiology. Cells were grown in M9 glucose medium supplemented with appropriate inducers at different concentrations (CHPA for CprK1/P_{DB3}; IPTG for $LacI^q$/*Ptrc*). The specific growth rate (μ) was calculated during the exponential growth phase.

In *P. putida*, the CprK1/P_{DB3} system was more precisely regulated than $LacI^q$/*Ptrc* **(Fig. 17b, 17c)**; the growth rate decreased when the inducer concentration was increased, which was far more evident in the case of pSnox. Moreover, P_{DB3} basal activity was lower than that of *Ptrc*; this regulation is another important advantage of the system. We also compared both expression systems in *E. coli* BW25113. Results indicated that, at difference from *P. putida*, *nox* overexpression provoked a severe decrease in the specific growth rate (**Fig. 17d, 17e)**. Three factors could explain this behavior. First, *E. coli* might be sensitive to high CHPA concentrations; second, *P. putida* is generally able to regenerate NADH efficiently, which could justify the more rapid growth rates. Finally, the expression systems tested behaved differently in both strains; as anticipated, $LacI^q$/*Ptrc* regulated *E. coli* correctly, while CprK1/P_{DB3} was functional in both strainsl *E. coli* and *P. putida*. In summary, we engineered an expression device formatted as a pSEVA standard vector; we validated its function in *E. coli* and *P. putida* using two approaches, reporter and physiology assays.

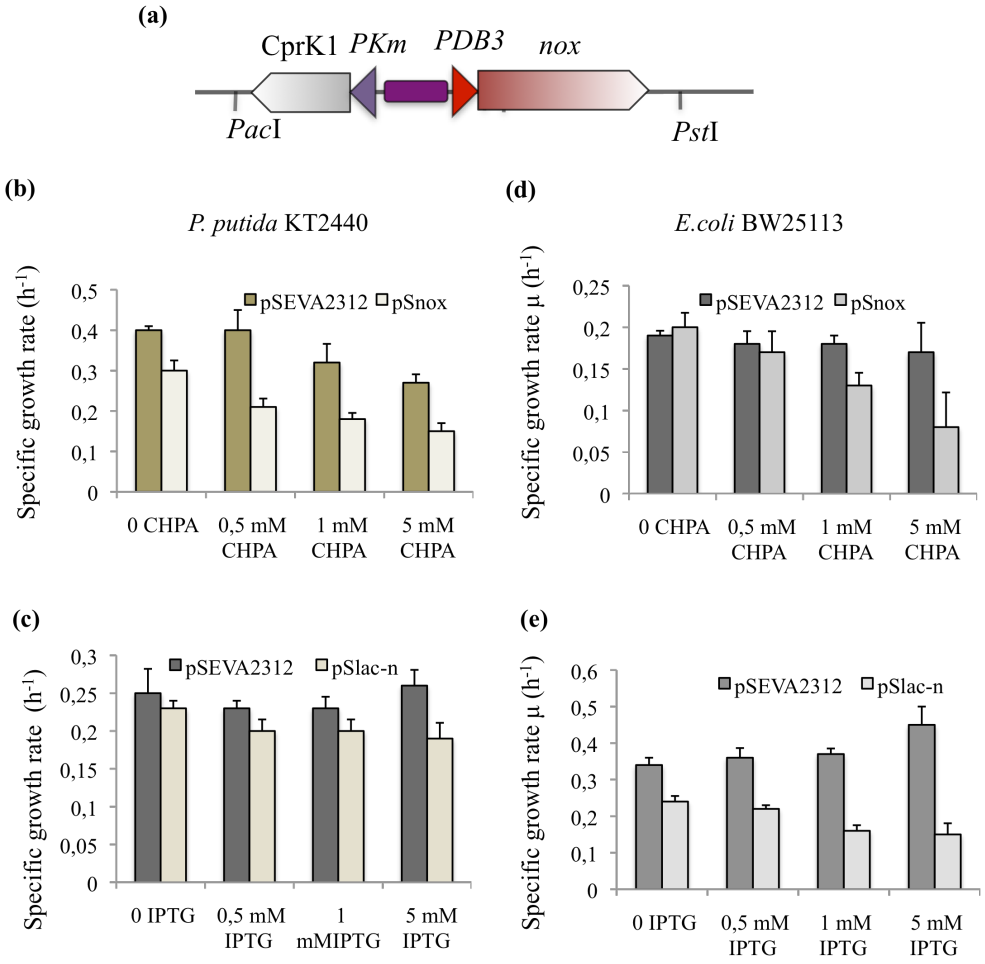

Fig. 17. Specific growth rates of *P. putida* KT2440 and *E. coli* BW25113 strains on glucose with different Nox activity levels. *P. putida* KT2440 strains bearing pSnox **(a)** were grown in M9 glucose with CHPA at different concentrations, to induce *PDB3* **(b)**. The *lacIq/Ptrc* plasmid, which controls *nox* expression (pSlac-n, lab collection) was used as positive control **(c);** pSEVA2312 (plasmid without *nox*) was used as negative control. *E. coli* BW25113 cells were transformed with pSnox, pSlac-n and pSEVA2312 plasmids. Strains were grown on M9 glucose medium and with inducers CHPA **(d)** and IPTG **(e).** Specific growth rate was calculated during the exponential growth phase, identified as the linear region in plot of the ln(CDW) *vs.* time. Bars show mean values ± standard deviation for each parameter of triplicate measurements from at least three independent experiments.

Chapter II

The dynamics between regulator and promoter in single cells: the case of XylR-*Pu* in *Pseudomonas putida*

Part of this section is in preparation as:

Guantes R, Benedetti I. and de Lorenzo V. Growth control of gene expression noise in the TOL network.

Background

Cells react to the environment through gene regulatory networks that process exogenous or endogenous inputs and determine survival of the organism in changing conditions (Silva-Rocha and de Lorenzo, 2010). This is the case of transcription factors, which sense the signal and trigger or inhibit transcription of a specific DNA sequence into mRNA (Calles and de Lorenzo, 2013). This interplay between inputs, regulators, RNA polymerase and target DNA (promoter) thus originates changes in regulatory complexity that determine the performance of a cell in a fluctuating environment (Calles and de Lorenzo, 2013; de Las Heras et al., 2010; Silva-Rocha and de Lorenzo, 2010).

In this chapter, we examine some properties of the regulatory node XylR-*Pu*, part of the TOL plasmid network of *Pseudomonas putida* mt-2, which is involved in the degradation of aromatic compounds such as *m*-xylene or toluene. XylR is the regulator (Ntrc family (Tropel and van der Meer, 2004) that responds to external effectors, in this case an aromatic molecule, and once activated binds its target promoter *Pu*. This process causes transcription of genes of the so-called upper pathway, which encodes enzymes necessary to convert the initial compound (toluene) into intermediates (3-MBz) that will enter the lower pathway to be metabolized **(Fig. 18)** (Calles and de Lorenzo, 2013; Ramos et al., 1997; Silva-Rocha and de Lorenzo, 2012b). Although the molecular mechanisms underlying these processes have been described extensively, we have little information about the dynamics of the regulatory device in single cells. Recent studies analyzed the behavior of TOL promoters in *P. putida*, and demonstrated that they showed a bimodality in which, on the appearance of a signal (e.g., *m*-xylene), the transcription state splits into two phenotypes (activated and non-activated (Nikel et al., 2013c; Silva-Rocha and de Lorenzo, 2013). Based on this background, we tested *Pu* behavior in several contexts, including change in regulator XylR concentrations and in the presence of distinct XylR effectors. We also analyzed XylR translation in single cells in a catabolic repression regime, focusing specifically on the role of the Crc protein in inhibition of XylR translation.

1. Experimental evidence of XylR as a limiting factor for *Pu* activity

XylR is the regulator that triggers *Pu*, the TOL pathway promoter (Fraile et al., 2001; Salto et al., 1998). This transcription factor also auto inhibits its own expression by repressing its promoter *Pr* (Ramos et al., 1997), which provokes release of a small amount of XylR protein. In natural conditions, XylR is thus present at low concentrations, which could explain in part the stochastic behavior of the *Pu* promoter (Nikel et al., 2013c; Silva-Rocha and de Lorenzo, 2012b, 2013). Indeed, TOL promoters such as *Pu* respond to inducers in a typical bimodal distribution, which might originate by limiting the amount of its regulator (Silva-Rocha and de Lorenzo, 2012b) In a recent study (Guantes et al., manuscript in preparation), simulations showed that in exponential growth phase, XylR overexpression implied a change in dynamics of the system, and specifically in a loss of *Pu* bimodality. To validate this model, we designed an experiment that consisted of gradually increasing XylR, with detailed analysis of *Pu* activity in single *P. putida* cells. We engineered the mini-Tn*7* pIB1 (see Material and Methods, **Table 2**) bearing the regulation cassette $LacI^q$/*Ptrc*-XylR (Perez-Martin and de Lorenzo, 1996) and inserted it into the chromosome of *Pseudomonas putida* KT2440 strain bearing a transcriptional fusion *Pu*→GFP (*P. putida* KT-IB1, see **Table 1**; **Fig. 19**).

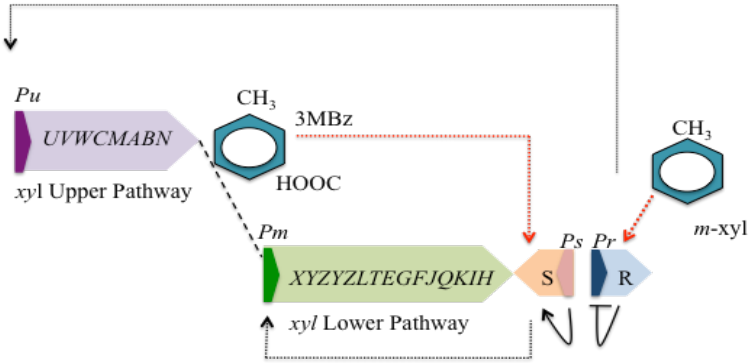

Fig. 18. Mechanism of XylR-*Pu* activation in the TOL network. The XylR regulator is expressed from the *Pr* promoter. When activated by *m*-xylene (*m*-xyl) (Velazquez et al., 2006), it triggers *Pu* expression while repressing *Pr*. *m*-xyl is converted to its intermediate 3-MBz, which activates the other regulator, XylS; this is turn triggers expression of the *lower* pathway from the *Pm* promoter. This pathway will metabolize 3-MBz into Tricarboxylic acid cycle (TCA) intermediates.

To control XylR expression, the *Ptrc* inducer IPTG was added to medium at different concentrations during cell growth, and *m*-xyl was added in the exponential phase to activate XylR. Post-induction GFP readout was acquired by flow cytometry once per hour over a 4-h period (**Fig. 20**). As IPTG concentration increased, *Pu* tended to lose its bimodality due to augmented XylR production. Compared to the control strain (*Pseudomonas putida* m/mt-2 *Pu*, (Silva-Rocha and de Lorenzo, 2012b), in *P. putida* KT-IB1 in the absence of IPTG, *Pu* seemed to have already lost its bimodality; this can be explained by the basal level of *Ptrc* promoter that enabled a larger number of XylR molecules than in wild type conditions. The shape of flow cytometry peaks (**Fig. 20**, column 1; no IPTG in medium) suggests cell-to-cell variability. In microscopy experiments, we confirmed that cell heterogeneity depends on XylR production. Once exposed to *m*-xyl vapors, *P. putida* cells showed greater heterogeneity (more GFP signal variation) in the absence of IPTG than when treated with 100 μM IPTG; **Fig**. **21a-21c** upper panels show *Pseudomonas putida* cells grown without IPTG, and lower panels represent the same strain with IPTG. Our experiments confirmed that the number of XylR copies in *P. putida* cells growing in exponential phase control *Pu* bimodal behavior in single cells.

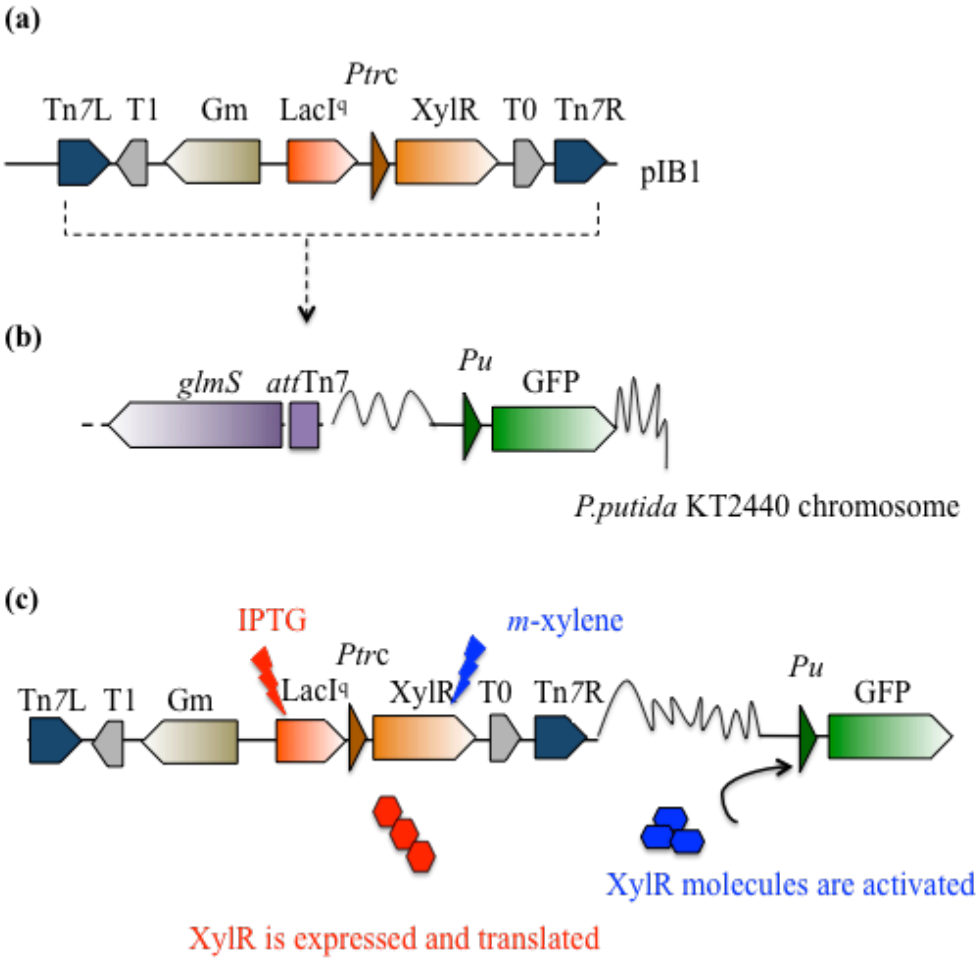

Fig. 19.Chromosomal insertion of pIB1 and activation of the system. (a) Transposable elements in pIB1 comprise two Tn*7* extremes (Tn*7*L and Tn*7*R, recognized by the transposase complex), two terminators (T1 and T0), the Gm resistance marker, and the LacI^q/*Ptrc* expression system, which controls XylR transcription. **(b)** This block is inserted into the chromosome of *P. putida* KT2440, which leads to transcriptional fusion of *Pu→GFP* into the genome. Tn*7* is inserted in 95% of cases at a specific *att*Tn*7* site, upstream of the 5' UTR of the conserved gene *glmS*. **(c)** IPTG induces LacI^q/*Ptrc* that controls XylR molecular transcripts; when *m*-xyl is added to medium, it activates XylR and triggers GFP expression from the *Pu* promoter.

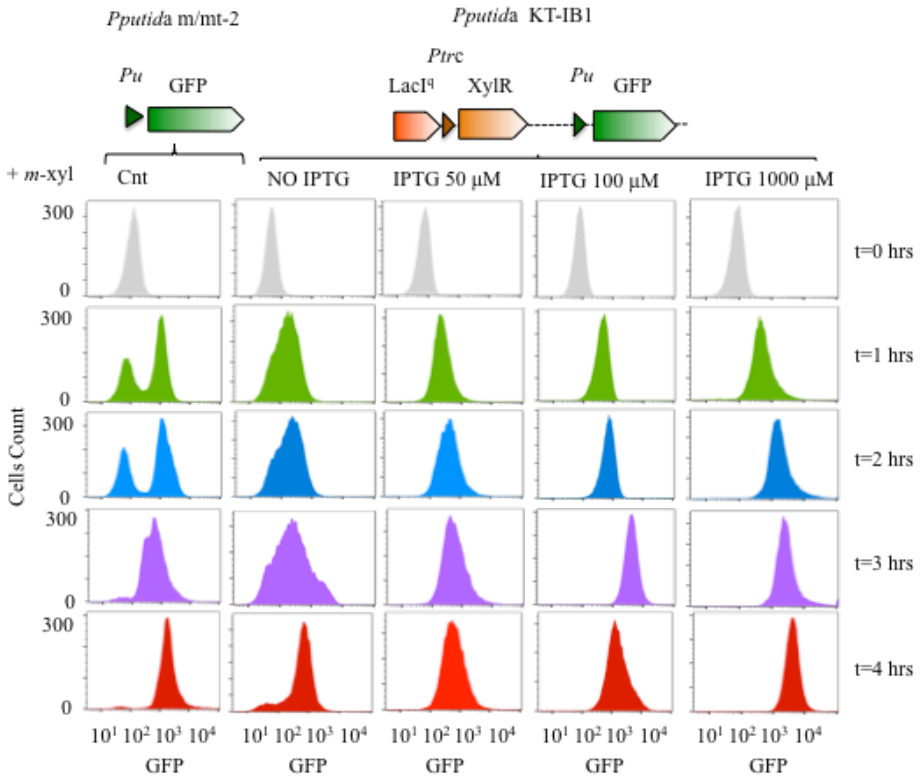

Fig. 20. Single cell analysis in *P. putida* KT-IB1 strains. Cells grown overnight were diluted 1/50 in M9 succinate medium supplemented with IPTG at different concentrations. At mid-exponential phase, cells were exposed to *m*-xyl vapors. Samples were collected at 1-h intervals and stored on ice until analysis by flow cytometry. Untreated cells were used as controls. For each assay, 20,000 cells were analyzed. P. putida m/mt-2 *Pu*-GFP was used as control strain for *Pu* activation profile.

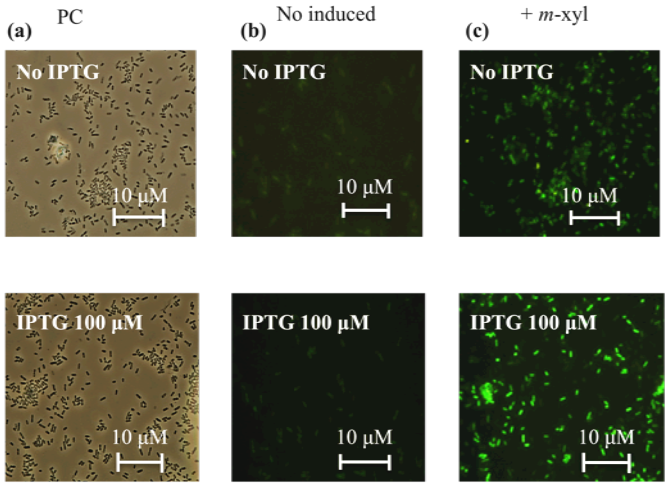

Fig. 21. Visualization of *Pu* activity in single cells of *P. putida* KT-IB1. After overnight growth, *P. putida* cells bearing *Pu*→*GFP* and LacI^q/*Ptrc* chromosomal insertions were diluted 1/50 and grown in M9 succinate medium supplemented with different IPTG concentrations to mid-exponential phase. *m*-xyl was added as a XylR inducer; at 4 h post-induction, cells were analyzed by **(a)**, phase contrast microscopy (PC) or fluorescence microscopy for **(b)** non-induced strains and **(c)** induced strains (GFP signal corresponds to *Pu* activity).

2. XylR effector specificity stimulates a variable response of *Pu* promoter in single cells

XylR protein is the main regulator of transcriptional control of TOL catabolic pathways (Marques et al., 1994; Marques and Ramos, 1993; Ramos et al., 1997). The *xylr* gene is expressed constitutively from two tandem promoters, but is activated and can induce its target promoter *Pu* only when it binds an effector (*m*-xyl) (de Lorenzo et al., 1993; Marques and Ramos, 1993; Ramos et al., 1997). This regulator recognizes a wide variety of inducers including toluenes, benzyl alcohols, bi-substituted aromatic rings such as *p*-nitrotoluene as well as tri-substituted rings such as 2,6-dichlorotoluene (Abril et al., 1991; Abril et al., 1989; Fernandez et al., 2009; Skarfstad et al., 2000). Previous studies analyzed the XylR effector profile and reported different *Pu* induction kinetics, depending on the effector that bound its regulator XylR (Abril et al., 1989). This difference in *Pu* activation indicated that the nature of the substituent on the aromatic ring of the inducer is critical for a productive or non-productive XylR state (Fernandez et al., 2009; Salto et al., 1998). On the basis of these findings, we analyzed *Pu* induction using distinct XylR activators to delineate a profile of effector specificity in single cells; in other words, what defines a good or bad effector.

We obtained a *P. putida* mt-2 strain with a *Pu*→GFP transcriptional fusion inserted into the chromosome (see Material and Methods) by engineering a mini-Tn*7* bearing *Pu→GFP*; we cloned the *gfp* gene into pTn*7*-M as a *Hind*II/*PstI* fragment. We then excised *Pu* from the pEZ9 plasmid (de Lorenzo et al., 1991) as *Eco*RI/*Bam*HI and inserted it into intermediate pTn*7*-GFP. The resulting plasmid, termed pTn7-*Pu*GFP, was mobilized by triparental mating to *P. putida* wild type strain *mt-2*. To analyze the *Pu* promoter, we grew cells in M9 citrate medium overnight, diluted cultures 1/50 in the same medium, and allowed them to grow to mid-exponential phase. Strains were exposed separately to vapors of four different inducers: *m*-xyl, 1,3,5-trimethylbenzene (1,3,5-tMBe), 2,6-dichlorotoluene (2,5-DCT) and benzaldheyde (BA). We selected these compounds based on the effectors reported by Abril et al., who analyzed *Pu* activity in response to a wide spectrum of inducers (Abril et al., 1989). After effector addition, we examined

samples by flow cytometry, with hourly acquisition of the GFP signal. **Figure 22a-d** show the distribution of cells induced by different induction regimes; *m*-xyl was the optimal inducer, 1,3,5-tMBe and 2,6-DCT acted as suboptimal effectors, while BA showed no notable effect (also as it was consumed by *P. putida* (Ramos et al., 1997). In all cases, when we observed cell distribution, we distinguished an increase in noise after inducers were added; *Pu* bimodality was maintained, which indicates that its activity was conditioned only by the inducer, independently of its chemical structure. Induction profiles confirmed that each effector provoked a different intensity of *Pu* response, especially in early exponential growth phase **(Fig. 23a-23d)**. Regarding the nature of the regulator/inducer interaction, we propose two possibilities, [i] XylR binds its effector but not always with the same efficiency (**Fig. 24a**) or [ii] XylR always binds its effector but activates *Pu* with different frequencies (**Fig. 24b**).

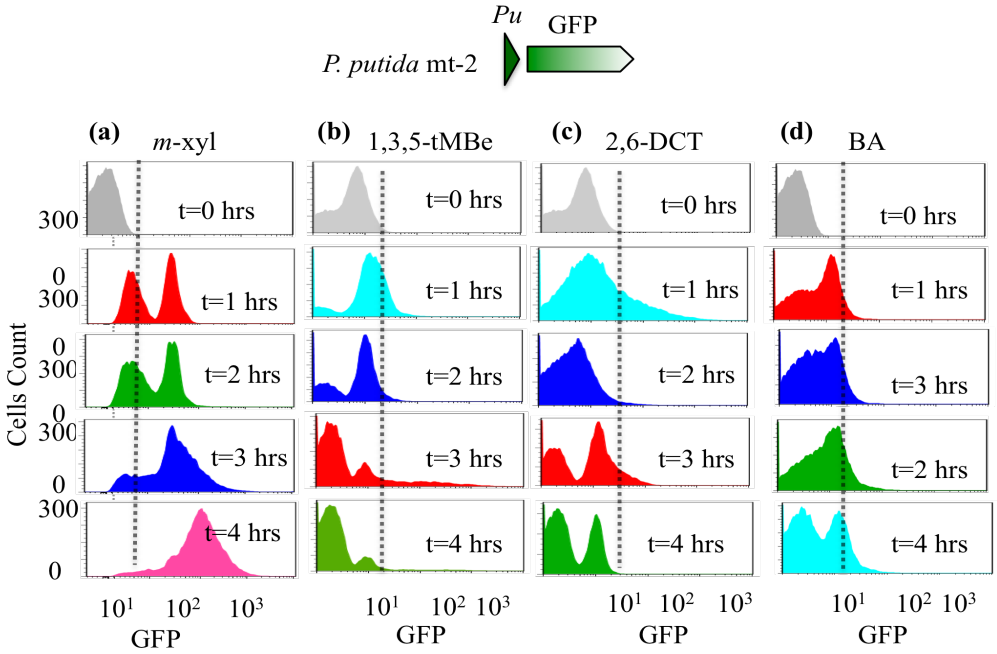

Fig. 22. Inducer profiles in single cells of *P. putida* mt-2 Pu-GFP. Cells grown overnight were diluted 1/50 and cultured in minimal medium to early exponential phase. Strains were then exposed to the inducers **(a)** *m*-xyl **(b)** 1,3,5-tMBe, **(c)** 2,6-DCT and **(d)** BA; samples were analyzed by flow cytometry at 1-h intervals. Untreated cells were used as negative controls; 20,000 events were counted for each assay.

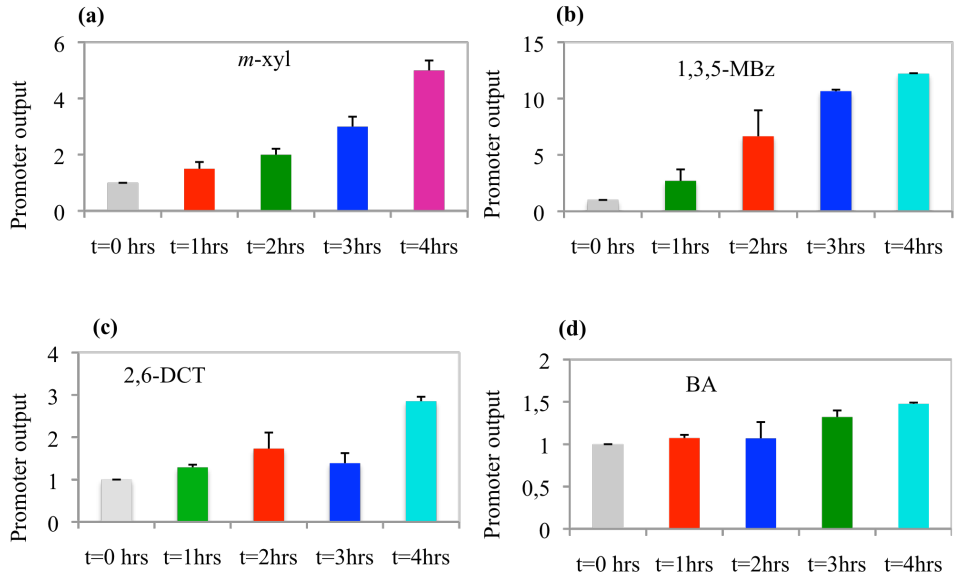

Fig. 23. *Pu* induction with different XylR effectors. Induction profile of *P. putida* mt-2 *Pu*-GFP in response to **(a)** *m*-xyl, b) 1,3,5-tMBe, **(c)** 2,6-DCT or **(d)** BA; profiles were calculated by normalizing average fluorescence levels of induced populations to fluorescence levels of untreated control samples. Bars show mean values ± standard deviation for each parameter in triplicate measurements from at least four independent experiments.

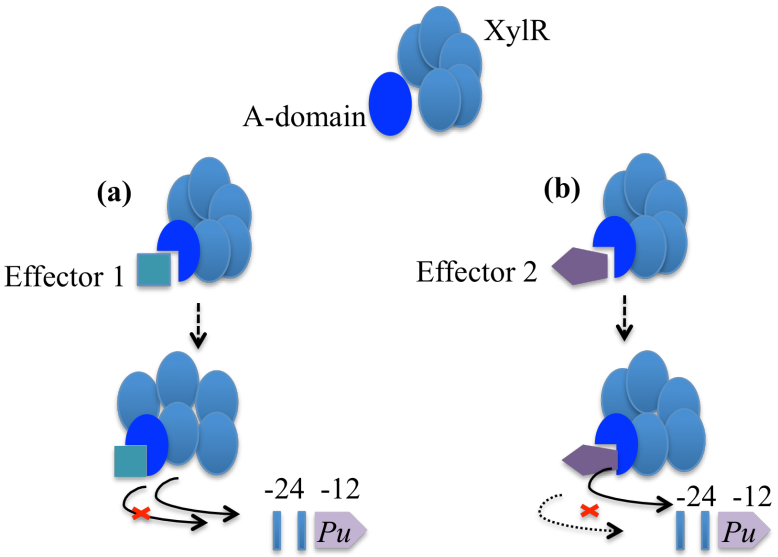

Fig. 24. Proposed mechanisms of XylR activation by suboptimal inducers. XylR is a hexamer and its A domain interacts with a wide variety of inducers. A suboptimal effector can activate the *Pu*-XylR network in one of two ways: (a) the molecule binds the regulator but the complex rarely activates the target promoter, or (b) the effector does not bind perfectly to the regulator, which thus that cannot adopt the necessary conformation to trigger *Pu* expression.

3. The Crc effect on XylR translation: monitoring protein production in single *Pseudomonas putida* cells in a catabolite repression regime

Crc protein is a global regulator involved in catabolite repression in *Pseudomonas* for the hierarchical and sequential use of a carbon source, when the growth medium is rich in nutrients (Hernandez-Arranz et al., 2013; Moreno and Rojo, 2008). It was suggested that Crc binds an A-rich motif AAnAAnAA (CA motif) within or adjacent to the ribosome binding site (RBS) of several target mRNAs, thereby preventing their translation (Moreno et al., 2010; Moreno and Rojo, 2008). The role of Crc was recently revisited and Hfq was reported as an additional protein that facilitates Crc-mediated translation repression (Hernandez-Arranz et al., 2013; Madhushani et al., 2014). Hfq was also proposed as a principal regulator that binds the CA motif of several mRNAs in *Pseudomonas aeruginosa* and acts as translational repressor (Madhushani et al., 2014; Sonnleitner and Blasi, 2014). Crc appears to contribute to Hfq-mediated repression during CCR, however, and it might interact with the regulator to improve Hfq specificity for A-rich sequences (Sonnleitner and Blasi, 2014).

Given these results, we analyzed Crc effects on XylR translation *in vivo*, and quantified its production in single cells of *Pseudomonas putida*. We designed translational fusions between XylR and msfGFP; we coupled first 10 amino acids of XylR to the reporter (see Material and Methods) and cloned the resulting constructs (XylR1a –bearing the *Pr* promoter– and XylR1b –bearing the P_{EM7} promoter) into the mini-Tn*7* vector, to obtain monocopy systems to insert into the *P. putida* chromosome (**Fig. 25a**). We generated two other XylR versions in which the CA motif (Hfq-Crc binding site) was mutated (see complete sequence in **Table 4**), which resulted in XylR2a and XylR2b constructs (**Fig. 25b**). Both XylR-fusion versions were mobilized into the genome of two *P. putida* strains, the wild type *P. putida* KT2440 and the *crc* mutant *P. putida* KT2442 (Moreno et al., 2010). To quantify XylR translation, *P. putida* cells of wild type and Crc mutant were grown overnight in LB medium (rich medium) at 30ºC, diluted 1/100 in the same medium and allowed to grow (2 h, 30ºC) to early exponential phase (OD_{600} 0.2). Samples

were collected every 30 min for 3 h (until stationary phase) and analyzed by flow cytometry. **Figure 26a, 26b** show absolute values of GFP expressed in *P. putida* strains bearing XylR1a and XylR1b. As predicted, the fluorescence signal was higher in Crc mutants than in wild type strains in the exponential phase, and the Crc effect on XylR translation was more evident when the *PEM7* promoter controlled transcription of the fused protein. When we used XylR with the Hfq/Crc-insensitive site, the fluorescence signal was much lower and the protein did not appear to translate adequately (**Fig. 26c**, **26d**). Deletion this site, located in the Shine-Dalgarno sequence (see **Fig. 25b**), probably affected protein translation. Change calculated as the ratio of GFP values for mutant and wild type strains indicated that in *P. putida* strains with an Hfq/Crc-insensitive binding site (KT-XylR1b and KT-XylR2b), catabolite repression was partially relieved **(Fig. 27a, 27b)**. In summary, we examined the Crc protein effect on XylR translation in single cells of *P. putida* and confirmed that Crc participates in the control of XylR production in rich medium. The lower level of Crc inhibition than anticipated nonetheless suggests that other factors contribute to the complexity of the TOL regulatory network.

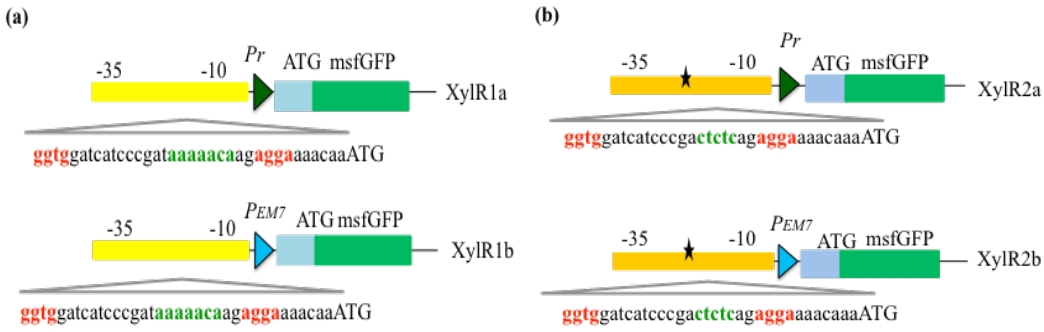

Fig. 25. Structural organization of XylR::msfGFP-based systems. Relevant genetic features of XylR::msfGFP translational fusions. Sequences for the putative Hfq/Crc binding site (green) and -35 and -10 (red) are indicated (for details, see complete sequences in **Table 4**). Fragments were inserted into *P. putida* KT2440 and *P. putida* Crc$^-$. **(a)** Hfq/Crc binding site inserted in the RBS region was maintained as in natural conditions (green) and expression of the XylR::msfGFP fusion was driven by *Pr* (XylR1a) and *PEM7* (XylR1b) promoters. **(b)** Hfq/Crc binding sequence (green) was mutated to render it insensitive; expression of XylR::GFP fusions were controlled by *Pr* (XylR2a) and *PEM7* promoters (XylR2b).

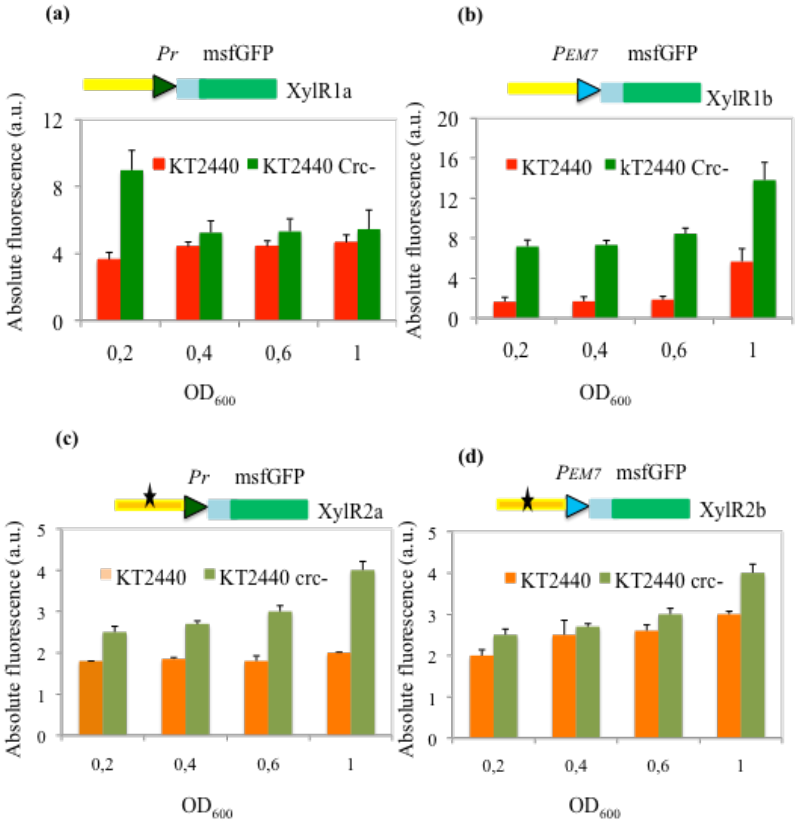

Fig. 26. XylR expression in a Crc variant of *P. putida* strains. Cells grown overnight were diluted 1/50 and cultured in LB to early exponential phase. Samples were collected every 15 min to the stationary phase (OD_{600} = 1.0) and analysed by flow cytometry. Fluorescence values for (a) XylR1a and (b) XylR1b in *P. putida* KT2440 and *P. putida* Crc-, or (c) XylR2a and (d) XylR2b in *P. putida* KT2440 and *P. putida* Crc mutant. Bars show mean values ± standard deviation of triplicate measurements for each parameter from at least four independent experiments.

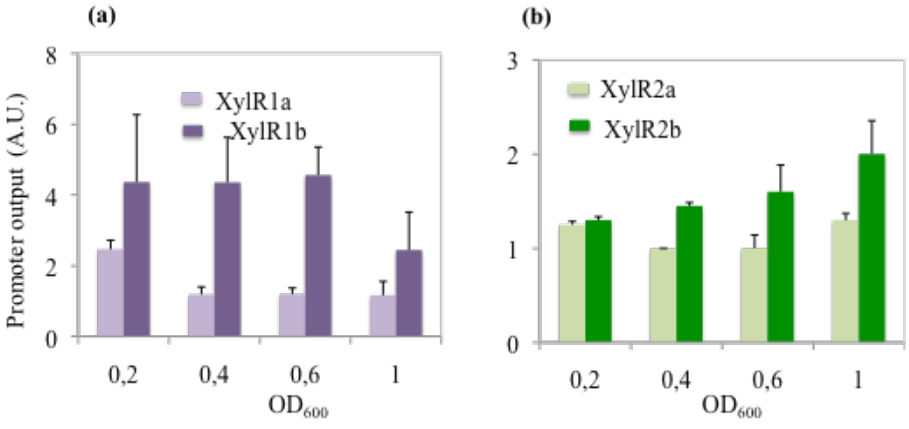

Fig. 27. Quantification of XylR translation in wild type and Crc mutant strains of *P. putida* KT2440. Profiles of each XylR variant were calculated by normalizing average fluorescence levels of Crc mutants to those of wild type populations. Bars show mean values ± standard deviation of triplicate measurements for each parameter from at least four independent experiments.

Chapter III

Engineering orthogonal expression systems for the design of catalytic biofilms with *Pseudomonas putida* KT2440

This chapter is in preparation as manuscript:

Benedetti I., de Lorenzo V. and Nikel P.I.: Engineering orthogonal expression systems for the design of catalytic biofilms with *Pseudomonas putida* KT2440

Background

Pseudomonas putida is a Gram-negative bacterium that colonizes the soil ubiquitously and metabolizes a broad range of natural and synthetic organic compounds (Arce-Rodriguez et al., 2012; Paez-Espino et al., 2014; Timmis, 2002). Because of their ability to degrade pollutants, *P. putida* strains are extensively studied for a number of industrial and environmental applications (Martinez-Garcia et al., 2014a). This bacterium colonizes soils (roots and soil particles), but produces poor biofilms that are usually thin, weak and discontinuous (D'Alvise et al., 2010; Fazli et al., 2014). The ability to make biofilm is nonetheless an important requisite for many biotechnological applications (industrial, medical, environmental), which increases interest in understanding the genetic elements, molecular mechanisms and environmental cues that regulate biofilm development in *P. putida* (Kuchma and O'Toole, 2000; O'Toole et al., 2000; Tolker-Nielsen et al., 2000). Some analyses of mass sequences of bacterial genomes detected an abundance of proteins with GGDEF and EAL domains, which are involved in turnover of the secondary messenger cyclic-diguanosine-monophosphate (c-di-GMP) (Hengge, 2009; Morgan et al., 2014). The GGDEF domain is responsible for formation of this molecule through diguanylate cyclase activity, whereas EAL is a phosphodiesterase domain that degrades c-di-GMP (Gjermansen et al., 2006; Simm et al., 2004). Recent findings show that c-di-GMP acts as universal second messenger that directs the transition from sessility to motility, and that proteins with GGDEF/EAL domains regulate cell adhesiveness and biofilm formation in a wide range of bacteria, including *P. putida* (Osterberg et al., 2013; Simm et al., 2004). The elevated cyclase activity in *P.putida* induces synthesis of biofilm matrix material, whereas elevated phosphodiesterase activity leads to dispersal of formed biofilm (Gjermansen et al., 2006; Morgan et al., 2014). In this section, we describe the standardized ChnR/*PchB* expression system we used to control production of cyclase and phosphodiesterase proteins to enhance biofilm formation in *P. putida*. In addition, we evaluated our system by designing catalytic biofilms for degrading halo compounds.

3.1 Design of standardized ChnR/*PchnB*-based systems

ChnR is the regulator that triggers transcription of the gene cluster responsible for cyclohexanol oxidation in *Acinetobacter* spp. and activates expression of a cyclohexanone 1,2-monooxydase (*chnB*) and a cyclohexanol dehydrogenase (*chnA*) (Iwaki et al., 1999). This gene cluster also enables *Acinetobacter* spp. to use several cyclic alcohols and alkenes as their sole carbon source (Steigedal and Valla, 2008). ChnR is similar to other bacterial transcription regulators such as AraC and XylS, and responds to several inducers (e.g., cyclohexanone; (Donoghue and Trudgill, 1975). We used this mechanism to design a fragment bearing the complete ChnR sequence and its target promoter *PchnB*, and edited it to obtain a standardized SEVA cargo. In addition, we inserted a linker that comprises a tir element, a strong P_{Km} promoter that drives ChnR expression, and a spacer sequence to insulate regulator expression from the rest of the gene (see **Table 4**; Materials and Methods). We assembled the expression system as a *Pac*I/*Avr*II fragment into pSEVA231 (Silva-Rocha et al., 2013) and denominated the resulting plasmid pSEVA2311. To validate the system, we cloned the reporter msfGFP into pSEVA2311 as a *Hind*III/*Spe*I segment and generated pSEVA2311-M (**Fig. 28a**).

3.2 Validation of pSEVA2311

We evaluated the activity of the expression system by quantifying GFP signal by flow cytometry. *E. coli* cells were grown in various media (LB, M9 with glucose or M9 with succinate as typical glycolytic and gluconeogenic carbon sources) at 37ºC overnight, then diluted 1/50 and allowed to grow to mid-exponential phase (OD_{600} = 0.4). We then added 1.0 mM cyclohexanone (inducer) and we removed a sample every hour for 5 h for flow cytometry. We compared *PchnB* activity in the three media; **Fig. 28b** shows GFP expression at times of induction. The cells showed a graded behavior and no subpopulations were detected; *PchnB* appeared to be very sensitive to the inducer and had a low basal activity level (**Fig. 28c**). Results were similar in LB and M9 with succinate, which confirmed system robustness and its versatility in different growth conditions.

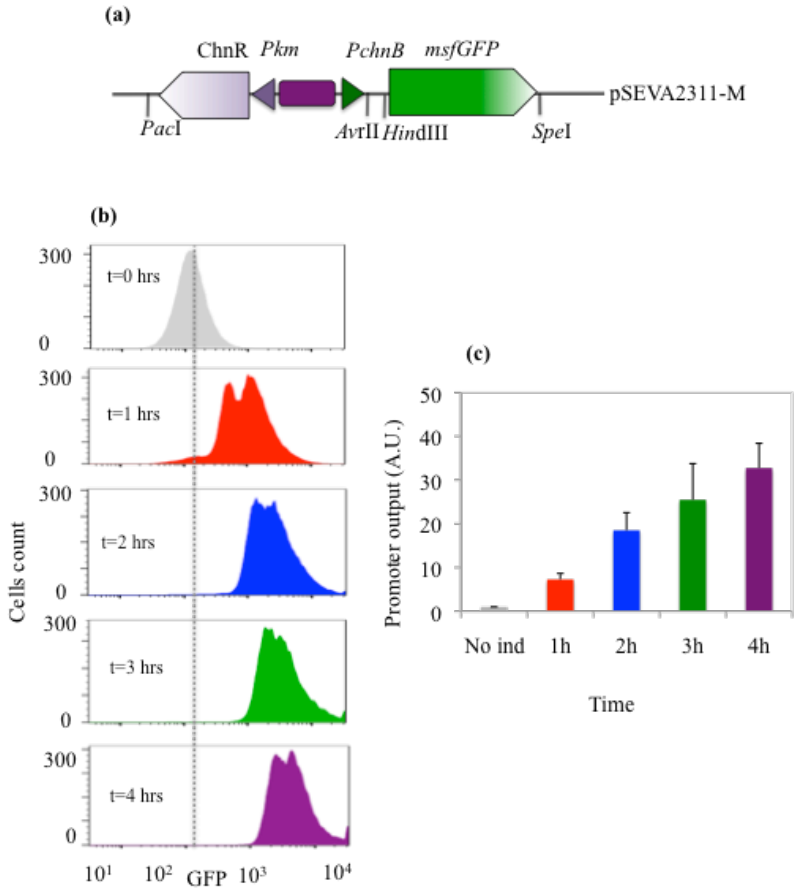

Fig. 28. Structural organization and validation of pSEVA2311. (a) Functional elements of the plasmid comprise [i] the positive regulator ChnR, [ii] a strong promoter that drives ChnR expression, [iii] a spacer sequence, [iv] the *PchnB* promoter activated by ChnR, and [v] the msfGFP reporter. **(b)** To validate the system, *E. coli* cells grown overnight were diluted 1/50 and allow to grow to mid-exponential phase. Cyclohexanone (1.0 mM) was added and samples were analyzed by flow cytometry at 1-h intervals, **(c)** Induction profile of *E. coli* pSEVA2311-M strain in response to 1.0 mM cyclohexanone; promoter output values were calculated by normalizing average fluorescence levels of induced populations to those of the untreated control samples. For each assay, 20,000 cells were analyzed. Bars show mean ± standard deviation of triplicate measurements from at least four independent experiments.

3.3 Engineering a new expression system to enhance biofilm formation in *P. putida* KT2440

Pseudomonas putida KT2440 is usually a poor biofilm former, which can be a disadvantage for biotechnological applications. To improve biofilm formation ability in *P. putida*, we modified expression of a guanylate cyclase gene by cloning the *yedQ* gene into pSEVA2311 as an *Avr*II/*Eco*RI fragment in *E. coli*; and termed

the resulting plasmid pS-edQ (**Fig**. **29a**). As controls, we used pSEVA2311 and the same version bearing the phosphodiesterase domain *yhjH*, which was cloned as a *Sac*I/*Bam*HI fragment into pSEVA2311 to yield a construct called pS-hjH (**Fig. 30a**). We transferred the plasmids (pSEVA2311, pS-edQ, pS-hjH) into the *P. putida* KT2440 strain by triparental mating and confirmed their presence by antibiotic selection and digestion of plasmid DNA with appropriate restriction enzymes. To test the function of the new constructs, we quantified biofilm formation in the recombinants. As described, cells carrying different plasmids were grown overnight diluted in two media (glucose or succinate) to OD_{600} of 0.1, and then divided in four multi-well plates. In the first, cells were grown in media with no inducer; the other three cultures were exposed to varying concentrations of cyclohexanone. Plates were allowed to grow at 30ºC for 24 h without agitation. Biofilm formation was quantified after 24 h static incubation using a crystal violet assay (see Material and Methods, (O'Toole and Kolter, 1998). The results indicated that strains bearing pS-edQ formed more biofilm than controls (Fig. **29b, 29c**) and that the level of biofilm production was proportional to inducer concentration. When we measured biofilm formation in strains bearing pS-hjH (phosphodiesterase domain), the opposite effect was observed; as the inducer concentration increased (corresponding to higher phosphodiesterase activity), strains produced less biofilm. We introduced an additional control, in which phosphodiesterase expression was controlled by the $LacI^q$/*Ptrc* system (pS-lacH plasmid). *P. putida* KT2440 biofilm decreased at higher cyclohexanone concentrations (**Fig. 30b, 30c**). Comparison of the two expression systems showed lower basal expression of ChnR/*PchnB* than $LacI^q$/*Ptrc*, again demonstrating its great versatility as a regulated expression system.

To analyze the biofilm by microscopy, we first introduced plasmids pS-edQ and pSEVA2311 into *P. putida* Δall-Φ by triparental mating. This is a *P. putida* KT2440 variant strain that does not carry any prophages and is thus more robust and viable (Martinez-Garcia et al., 2014a). The same strain was chromosomally marked with GFP, whose expression was driven by a constitutive promoter. For sample preparation, a glass coverslip was placed into 5 ml of M9 glucose medium

supplemented with 0.5 mM cyclohexanone and inoculated with culture to OD600 = 0.05 (see Material and Methods). After 48 h, coverslips were removed and prepared for microscopy analysis. **Figure 31a** shows phase contrast images, and **Fig. 31b** shows the same strains visualized in the fluorescence channel. Overproduction of cyclase, whose expression is controlled by the ChnR/*PchnB* system, induced synthesis of more biofilm matrix material than the control strain.

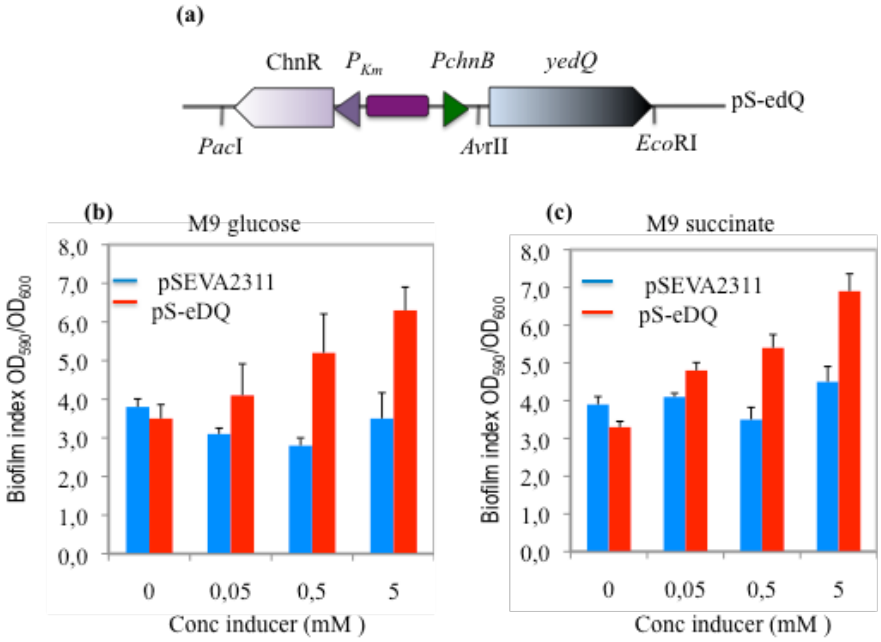

Fig. 29. Biofilm quantification in P. *putida* KT2440 strains bearing the cyclase gene regulated by the ChnR/*PChnB* system. **(a)** Scheme showing genetic elements in the pS-edQ plasmid: the cyclase gene (*yedQ*) was cloned into pSEVA2311 under the control of the ChnR/*PchnB* system. When cyclohexanone is added to growth media, it induces ChnR, which regulates *PchnB* activity; this in turn triggers cyclase gene transcription. **(b)** Biofilm formation in multi-well plates of *P. putida* KT2440 with pS-edQ and pSEVA2311 as control. Cells were grown in M9 media for 24 h without agitation, and supplemented with various concentrations of cyclohexanone. Values were obtained by normalizing crystal violet absorbance (590 nm) with the initial value at OD_{600}. Bars show mean ± standard deviation of triplicate measurements from at least three independent experiments.

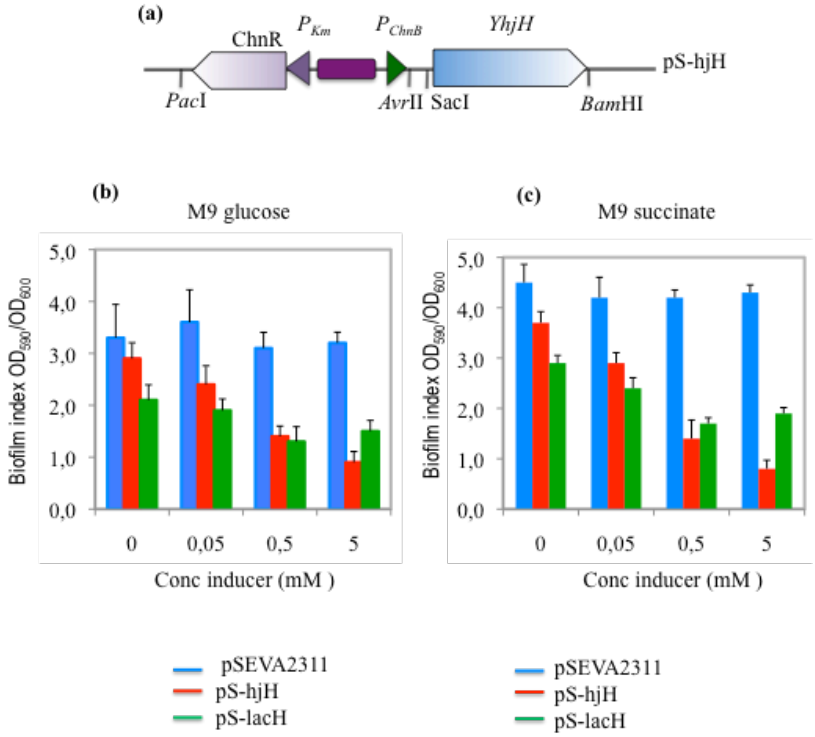

Fig. 30. Biofilm quantification in *P. putida* KT2440 strain carrying the phosphodiesterase gene regulated by the ChnR/*PchnB* system. (a) Scheme showing the genetic elements included in the pS-hjH plasmid. Phosphodiesterase (yhjH) was cloned into pSEVA2311 under the control of the ChnR/*PchnB* system. Cyclohexanone addition to growth medium produces effects as detailed in **Fig. 29b**. **(b)** Biofilm formation in multi-well plates of *P. putida* KT2440 bearing pS-hjH, pSEVA2311 and pS-lacH (with the phosphodiesterase gene under the control of the $LacI^q$/*Ptrc* system) as an additional control. Cells were grown in M9 media for 24 h (succinate or glucose) without agitation, then supplemented with various cyclohexanone concentrations. Values were obtained by normalizing crystal violet absorbance (590 nm) with the initial value at OD_{600}. Bars show mean ± standard deviation of triplicate measurements from at least three independent experiments.

3.4 Design of a functional catalytic biofilm

Interest exploiting robust catalysts for biotechnological application has grown in recent years. A number of studies found that use of biofilms could have an important role in biocatalyst processes, given their tolerance to environmental stress (organic solvents, heavy metals, antimicrobial compounds, toxic chemicals) compared to their planktonic counterparts (O'Toole and Kolter, 1998). We therefore designed a catalyst consisting of a *P. putida* KT2440 biofilm as a chassis for genes that allow degradation of synthetic haloalkanes such as 1-chlorobutane.

First, in *E. coli*, we cloned synthetic AHDO (alkyl halide degradation operon) as a *Kpn*I/*Hind*III fragment from pAHDO (Nikel and de Lorenzo, 2013a) into pSEVA4413, to obtain pSAHDO. This operon bears three genes (isolated in *Pseudomonas pavonaceae* 170) responsible for degrading several halo compounds. The structural organization of the synthetic operon is constituted by a hydrolytic haloalkane dehalogenase (*DhA* (Janssen, 2004; Poelarends et al., 1998) with broad substrate specificity, and the 3-chloroacrylic acid dehalogenase (*caaD*), composed of two subunits *CaaD1* and *CaaD2* (de Jong and Dijkstra, 2003; van de Pas et al., 1999) which catalyzed dechlorination of the target compound (**Fig**. **32a)**.

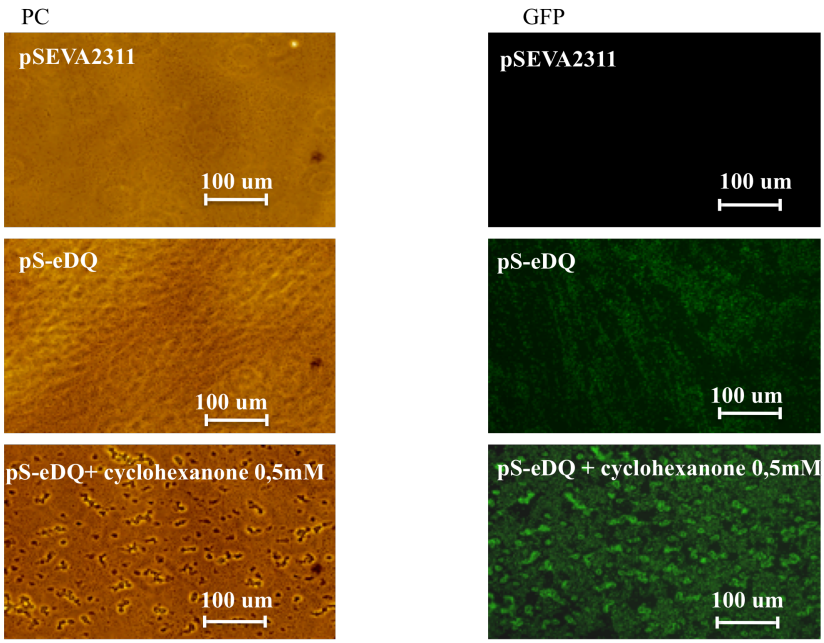

Fig. 31. Microscopy analysis of *P. putida* Δall-Φ with an overexpressed cyclase gene. Phase contrast **(a)** and fluorescent micrographs **(b)** of *P. putida* Δall-Φ biofilms. Cells bearing pS-edQ plasmid and pSEVA2311 (control) were grown in M9 glucose medium alone or with 0.5 mM cyclohexanone. A cover slide placed in inoculated medium constituted the surface for biofilm attachment. Images were taken after 48 h growth at room temperature without agitation.

The pSAHDO plasmid was mobilized to *P. putida* KT2440 bearing pS-edQ (biofilm-forming strain); as a control we used the empty plasmid pSEVA4413. For this assay (see Material and Methods), we prepared multi-well plates in which we

cultured cells bearing pSAHDO+ pS-edQ and pSEVA4413+ pS-edQ (control strain) in 200 μl M9 media (succinate or glucose) supplemented with 1.0 mM cyclohexanone. After 48 h (room temperature without agitation), we transferred 100 μl culture to another plate to obtain planktonic conditions; in the original plate, we discarded the remaining liquid to obtain biofilm conditions. Both plates were incubated overnight at 30ºC with 100 μl of a mixture of saturated 1-chlorobutane in water. After 24 h static incubation, we determined halide production spectrophotometrically (460 nm). We carried out *in vitro* screening for haloalkane dehalogenase activity in *P. putida* cells bearing AHDO and cyclase, in the biofilm and planktonic states (**Fig. 32b**, top and bottom, respectively). As anticipated, the highest enzyme activity was observed in multi-well plates with formed biofilm. We quantified *in vitro* the haloalkane dehalogenase activity in *P. putida* KT2440 expressing AHDO. Cells organized in biofilm showed from 2- to 4-fold higher dehalogenase activity than planktonic cultures, which indicated biofilm function as a catalyst (**Fig. 32c**).

In summary, we designed a pSEVA bearing the ChnR/*PchnB* heterologous expression system that responds to compounds such as cyclohexanone. We used this mechanism to control biofilm formation in *P. putida* KT2440, by insertion of the cyclase gene, which contributes to formation of biofilm matrix material. We also tested the catalytic abilities of these *P. putida* KT2440 biofilm-formers by introducing an organohalide biodegradation pathway. The results indicate a *P. putida* KT2440 able to produce more robust biofilm that are also catalytically active.

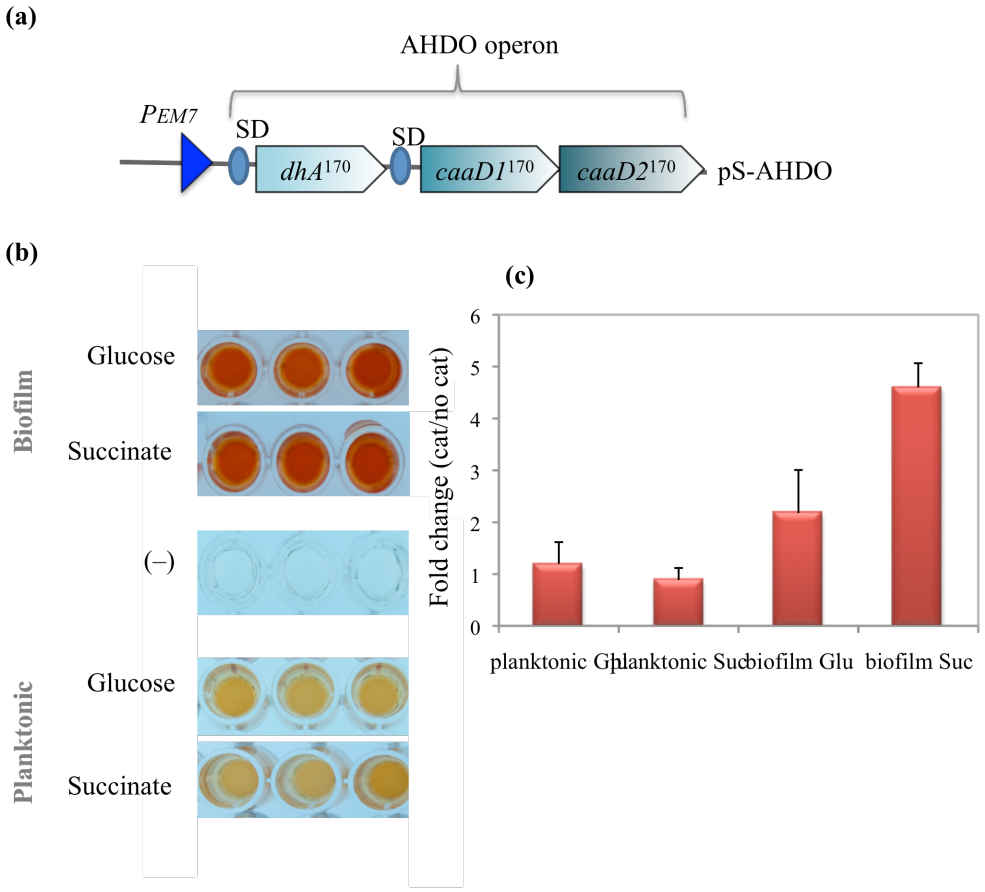

Fig. 32. Introduction of the alkyl halide degradation operon (*AHDO*) in *P. putida* KT2440. **(a)** Scheme showing the synthetic operon AHDO, in which *dhA170*, *caaD1*, *caaD2* genes were placed under the control of the *PEM7* promoter as a single transcriptional unit (Nikel and de Lorenzo, 2013a). **(b)** *In vitro* screening of haloalkane dehalogenase activity in *P. putida* KT2440 strains bearing an additional pS-edQ plasmid (which expresses cyclase under ChnR/*PchnB* control). Cells were grown in M9C (modified minimal medium with low chloride content) supplemented with glucose or succinate and cyclohexanone (ChnR inducer). After 48 h, samples were divided and prepared to obtain cells in planktonic or biofilm state. *P. putida* KT2440 bearing empty vector was included to evaluate background signal in control conditions. **(c)** *In vitro* quantification of relative haloalkane dehalogenase activity in *P. putida* KT2440 cells bearing an *AHDO* operon and cyclase gene, growing in planktonic or in biofilm conditions. Cyclase expression is controlled by the ChnR/*PchnB* system, which is induced by cyclohexanone. Changes were calculated by dividing values of enzyme activity obtained in biofilm by values obtained in planktonic cells. Bars show mean ± standard deviation of triplicate measurements from at least three independent experiments.

V. DISCUSSION

1. Design of standardized genetic tools for gene expression analysis in *Pseudomonas putida*

The lack of a common format for tools developed in different laboratories for similar purposes often impedes data comparison. It typically requires cloning of the same sequences into available vectors, which becomes an authentic bottleneck for engineering devices that involve parts from various origins (Arkin, 2008; Ellis et al., 2011; Muller and Arndt, 2012; Silva-Rocha et al., 2013). An immediate solution to this problem might consist of redesign of functional molecular tools, in a standardized format compatible with both old and new cloning and DNA assembly methods. A fundamental reference is currently the pSEVA platform, which collects a set of formatted vectors composed of synthetic, interchangeable and reusable functional modules. The work presented in this thesis follows this line of standardization, optimization and validation of genetic tools for Gram-negative bacteria, specifically *Pseudomonas putida* KT2440.

Demonstrating the function of a molecular tool is, however, insufficient to understand living systems. It is also necessary to show that the tool can be used to respond to biological questions. What are the consequences of phenotypic variability in specific environments? How do single cells behave in different genetic contexts? Given the importance of cell heterogeneity, what is the correct approach for its analysis? Can we efficiently modulate the state of live cells using a synthetic biology approach? This work contributes to answering these questions for comprehension of living systems.

Although we have a great variety of well-regulated alternative expression systems, we are still anchored to use of classical tools (such as $LacI^q$*/Ptrc),* which might not comply with all requisites for the resolution of molecular engineering bottlenecks. In Chapter I (section 1), we described a GFP-*luxCDABE* dual reporter system implanted in standardized broad host-range plasmids; its main utility is to analyze promoters in Gram-negative bacteria such as *E. coli* and *P. putida*. Using this system, we obtained information not only for whole population, but also for single cells in this population.

This exemplifies the potential of reporter assays, especially if distinct reporters are combined, to inspect gene expression levels (Benedetti I.M., 2012; Cox et al., 2010; Silva-Rocha and De Lorenzo, 2012a). We used pSEVA plasmids as a backbone to assemble and test expression systems that naturally respond to cyclo-alcohols or chlorinated compounds. In this case, we found an alternative to the more-exploited *LacI*^{q}*/Ptrc* system, which usually has high basal levels and impedes precise promoter analysis, especially in strains such as *P. putida*. Neither of our assembled systems, CprK1*/P*$_{DB3}$ and ChnR*/PchnB*, perturbed *E. coli* or *P. putida* physiology, and both were extremely sensitive to their own inducers (in the order of micromoles).

The plasmid approach is not always the best choice for understanding the dynamics of a specific regulatory network. To explore the precise behavior of single cells in a defined environment, we must eliminate all possible noise effects. Multi-copy systems such as plasmids could promote this type of effect and alter the analysis of cell-cell variability or stochastic phenomena (Becskei et al., 2001); alternative strategies must then be considered to obtain single copies of a host cell promoter or gene. The use of transposon vectors in molecular biology is not new, and the large number of available mini-transposon vectors sometimes complicates their application (which transposon should I use? What is the best format?). A number of standardized mini-transposons vectors is now available (Martinez-Garcia et al., 2011; Martinez-Garcia and de Lorenzo, 2012; Nikel and de Lorenzo, 2013b). Section 2 of Chapter I describes a set of mini-Tn*7* vectors whose format we standardized with SEVA architecture. We used these transposons for a series of experiments to calibrate synthetic promoters in *P. putida* (Chapter I, section 2), to analyze promoter activity in different genetic contexts (Chapter II, sections 1 and 2) and to monitor protein translation in single cells grown in a specific regime (Chapter II, section 3).

The SynPro library groups a number of synthetic promoters formatted with mini-Tn*7* vectors and that have a broad spectrum of intensities. Once integrated into the *P. putida* KT2440 chromosome, the promoters were quantified by their GFP output signal. **Figure 14** (Chapter I, section 2) illustrates an activity gradient of different

promoters and suggests that the majority have very high activity. It would thus be opportune to enrich our library with additional promoters that elicit lower intensity. Our future work will be directed to generation of a large collection of mono-inserting promoters and enable their use for constitutive, controlled expression of target genes.

2. What is the best approach? What is the best technique?

Both plasmids and transposon vectors contribute directly to engineering living organisms and to understanding their regulatory mechanisms. Plasmids serve fundamentally as platforms for cloning genes or expression systems, and are used for preliminary analyses. Transposons, in turn, are used mainly to obtain engineered systems similar to that of a natural organism. Chromosomal insertions can provoke malfunction of the inserted element, however, and in such a case a plasmid must be chosen.

With regard to technical approaches, we generally used flow cytometry to quantify the reporter signal. This technique was extremely powerful for analysis of cell heterogeneity and, indirectly, for understanding the regulatory dynamics of a biological system (Czechowska et al., 2008; Nikel et al., 2013c) . Microscopy has a dual role for these studies; first, it complements results obtained in quantitative experiments (flow cytometry or crystal violet assay) and second, it serves for analysis of single transcripts or for chromosomal localization (Kim J., doctoral thesis 2014). Finally, informatics empowers validation of the experiments through model predictions and simulations. This indicates the importance of integrating many approaches to obtain a solid understanding of living systems.

3. The dynamics of the XylR/*Pu* regulatory network in *Pseudomonas putida*

As discussed in the introduction, *Pseudomonas putida* KT2440 is an optimal chassis for engineering and assembling circuits with powerful potential for biotechnological applications. One of its most remarkable features of *P. putida* is its ability to use many aromatic compounds as an energy source, due to the presence of catabolic

genes that encode specific enzymes involved in the cleavage of aromatic rings (Nikel et al., 2014a; Silva-Rocha and de Lorenzo, 2013) .

XylR is perhaps one of the most promiscuous transcription factors of *P. putida* and responds to a wide range of effectors (Abril et al., 1991; Abril et al., 1989; Salto et al., 1998). It is still unclear how XylR interacts with these inducers to trigger *Pu* activity; we know that some compounds are better effectors than others, but we do not at the moment know why. We observed that, as described for bacterial populations (Abril et al., 1991; Salto et al., 1998), distinct XylR inducers also provoked a different response by the target promoter *Pu* in single cells. Moreover, independently of its chemical structure, the inducer stimulated bimodal *Pu* behavior during the initial phase of cell growth. It will be interesting to determine whether the inducers used by *Pu* have a similar effect on the other XylR-responsive promoter, *Ps* (which drives XylS transcription). A recent study redesigned the connectivity of the XylR/*Pu* regulatory network and found a decrease in XylR promiscuity (de Las Heras et al., 2012); this concept could also be applied to optimize the XylR response to other inducers.

As well as external factors, internal elements such as transcription factors help to define the conduct of a promoter. As shown in Chapter II (section 1), we found that few copies of XylR originated stochastic activation of the *Pu* promoter, and that this bimodality was lost when XylR concentration increased, even in the early phases of induction. Based on these results, we have clarified the role of XylR in *Pu* output, and found that response type and intensity are both generated by multi-factorial elements (environmental, genetic). This study contributes to our understanding of the intrinsic properties of *P. putida* regulatory networks, to allow rational design of engineered circuits.

4. The role of XylR in Crc-mediated catabolite repression

A separate discussion is warranted for the case of XylR and its controversial role in catabolite repression. Recent literature has revisited the role of Crc as a central

regulator of carbon uptake in a rich environment (Moreno et al., 2014; Sonnleitner and Blasi, 2014). Hfq was proposed as a fundamental factor that binds the A-rich DNA motif close to or within the RBS of many mRNAs, and it inhibits their translation. The best-supported hypothesis is that Crc cooperates with Hfq to bind DNA and improves Hfq specificity for the A-rich motif. In the case of XylR, Crc could also work with other factors. Our study showed that Crc has a less important role than previously thought as a controlling factor in XylR expression during CCR in single *P. putida* KT2440 cells.

5. Engineering *P. putida* K2440 to form active catalytic biofilm

In the final section of this study, we use standardized tools to engineer *P. putida* KT2440 as an efficient former of catalytic biofilm, thus furnishing an example of a direct application of synthetic biology to a biotechnological task. Using *P. putida* KT2440 as the host to express orthologous genes involved in halo compound degradation, we enhanced biofilm production by designing a standardized plasmid bearing the cyclase gene under the control of the cyclohexanone-responsive ChnR/*PchnB* expression system (Iwaki et al., 1999). The cyclase gene participates in the synthesis of c-diGMP, a multifunctional molecule implicated in matrix component production (Simm et al., 2004). Our results indicated that the modified strain was able to form much more biofilm than wild type *P. putida*, and that control of ChnR/*PchnB* activation modulated this ability.

In additional experiments, we used the phosphodiesterase gene, whose function is opposite that of cyclase, to comparing the levels of biofilm generation. Cells bearing the overexpressed phosphodiesterase gene behaved very similarly to wild type *P. putida* KT2440; this finding suggests, that in its natural environment, this strain overexpresses the enzyme that degrades c-diGMP and thus provokes formation of less matrix for surface adhesion.

To exploit the potential of neo-engineered *P. putida* KT2440, we designed a functional catalytic biofilm by inserting an additional standardized plasmid bearing

the synthetic operon AHDO (Nikel and de Lorenzo, 2013a), which codes for a dehalogenase that removes halogen groups from alkyl chains. The dehalogenase quantification assay (Fig. 32b) indicated that the catalytic biofilm formed by *P. putida* KT2440 produced more dehalogenase than the planktonic state (**Fig. 32b,** bottom), and thus shows potential for future applications. This study therefore offers a new perspective for the use of *P. putida* KT2440 as an exemplary chassis, and explores its use for novel biotechnological applications.

6. Final remarks

The results of this thesis highlight the importance of molecular tools for the study of diverse aspects of bacteria, from the creation of an optimized chassis, study of single cell variability, to direct applications for biotechnology. Our findings are the basis of future developments; for example, it will be important to complete the SynPro library with additional promoters with lower activity. We will also continue analysis of *nox* expression in *P. putida* and *E. coli* strains, to measure Nox activity *in vitro* as well as the NAD/NADH coefficient.

Comprehending the nature of the interaction between different effectors and XylR will be an interesting question, as will determining how this produces a specific response in *Pu*. *Pseudomonas putida* KT2440 as a biofilm former will be considered an indispensable tool for the exploration of still-unknown biofilm properties and regulation mechanisms. The world of synthetic biology offers continuous challenges: new techniques, tools, DNA approaches and practical applications are being developed daily. This study has provided a small but valuable contribution to this field.

VI. CONCLUSIONS

The results of this thesis have given rise to the following conclusions:

1. Genetic tools designed specifically for Gram-negative bacteria, plasmid- and transposon-based systems, are useful instruments for promoter analyses in populations and in single cells.

2. The increase in concentration of XylR regulator, i.e., the variation in XylR copies, suppresses the bimodality of its target promoter *Pu* in single *Pseudomonas putida* cells

3. The sub-optimal XylR effectors provoke bimodal response by the target promoter *Pu* in population as well as single *P. putida* cells

4. In our experimental conditions, Crc protein does not manifest to have a central role in inhibiting XylR translation in a catabolite repression of *Pu* in single *P. putida* cells

5. *P. putida* KT2440 can be engineered for biotechnological applications; for example, to produce a robust and catalytically active biofilm for degradation of halogenated compounds

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VIII. ANNEXES

Table 3. Oligonucleotides synthesized in this study for PCR reactions

Name (restriction site)	Sequence (5'-3')
5-GFP (*Pst*I)	GCGGCTGCAGGCATGCAGGAGGAAAAACATATG AGTAAAGG
3-GFP (*Hind*III)	GCGGAAGCTTCTATTTGTATAGTTCATCCATGCC
5-PB (*EcoR*I)	TGGATGAATTCGACAGTACCCTCC
3-PB (*Bam*HI)	GCGCGGATCCGGCCAGGGTCTCCCTTG
Tn7L	ATTAGCTTACGACGCTACACCC
Tn7R	CACAGCATAACTGGACTGATTTC
Ppu-glmS-Up	AGTCAGAGTTACGGAATTGTAGG
Ppu-glmS-Down	TTACGTGGCCGTGCTAAAGGG
Pm-5 *(Pac*I*)*	CGGCGGTTAATTAAGGTTTGATAGGGATAAGTCC
Pm-3 *(Avr*II*)*	CACATCCCTAGGTCTGTTGCATAAAGCCTAA
XylS-3 *(Pac*I*)*	CACACACACCTAGGGCGATGCCAACCCATC
5-XylR (*Eco*RI)	GCGGGAATTCCACATTAAAATAAGAGAACCGTG AACT
3-XylR (*Bam*HI)	GCGGGGATCCGCGATGCCAACCCATC
5-Pu (*Eco*RI)	GCGGAATTCGGAAAGCGCGATGAACC
3-Pu (*Bam*HI)	GCGCGGATCCCAACATTGAAGGGTCACCAC
5-CPRK1 (*Avr*II)	GCGCGCCTAGGGCTAGCATGGCTGTTGAAGGTTT GGGCAAG
3-CPRK1 (*Pac*I)	CGCGCTTAATTAACTAGTAATACGATGTTTGTTCA G
5-DB3 (*Nhe*I)	GCTAGCTTTTTCCTCCTTATAAAG
3-DB3 (*Avr*II)	CCTAGGCTCTCGACATTACGGG
5-ChnR (*Avr*II)	TTTTGCTAGCATGAGCACAGACAAAGCAAATAC
3-ChnR (*Pac*I)	TTTTTTAATTAATCAAAAAACAATAGAGGAGACT GAATTTTC
5-PEM7 (*Pac*I)	TAGCTTAATTAAAGTGAGCTCGTTGACAATTA
5- Pr *(Pac*I*)*	TATGTTAATTAATTTAATGTGGGCTGCTTGGT
3- Pr *(Avr*II*)*	CGGCCCCTAGGCAGCATTCCATCTGCCAC
3-msfGFP *(Bam*HI*)*	CGGGGATCCTTATTTGTAGAGTTCATCCATGCCGT GC
5-msfGFPT	AGCGGTGGCGGTGGCAGTAAAGGTG

3-XylRT	CACCTTTACTGCCACCGCCACCGCTCTGCATCTTG GGTTTGTATGTAAGC
5-YedQ (*Avr*II)	AAACCTAGGTTAGGAGGAAAAACACGTGCAGCA CGAGACAAAAATG
3-YeDQ (*Eco*RI)	AAAGAATTCTTAAGCGTTATCGCTCGC
5-YhjH (*Sac*I)	AAAGAGCTCTTAGGAGGAAAAACATATGATAAG GCAGGTTATCC
3-YhjH (*Bam*HI)	AAAGGATCCTTATAGCGCCAGAACCG

Table 4. DNA sequence of additional genetic elements used in this thesis

Name (restriction site)	Sequence (5'-3')
BCD2	CCTAGGGCCCAAGTTCACTTAAAAAGGAGATCAA CAATGAAAGCAATTTTCGTACTGAAACATCTTAA TCATGCTAAGGAGGTTTTCTAATGATCATGGGAA TTCAT
Spacer sequence	GGCAAAAAACATTATCCAGAACGGGAGTGCGCC TTGAGCGACACGAATTATGCAGTGATTTACGACC TGCACAGCCATACCACAGCTTCCGATGGCTGCCT GACGCCAGAAGCATTGGTGCACCGTGCAGTCGAT GATAAGCTGTCAAAC
PEM7	GGTTTAGTTCCTCACCTTGTCGTATTATACTATGC CGATATACTATGCCGATGATTAATTGTCAACAA
SynPro17	CGGAGTTGACAACACTCGAAAAGCCGAGTATAAT CAGATG
SynPro19	TCTCGTATTATAAAGTGTTTCCTGTTGATTGTCAA ATTGG
SynPro25	GCCCGTTGACATGACATGGTTTTGAGGGTATAAT GTGGCG
SynPro28	CTAGGTTGACATGGATATAATGTATGT
SynPro34	TAAGCGATTATACATGTGTTCGATTTCGATGTCA AATTAT
SynPro35	CCCCAAATTATAATTCTAAACATCACGCATGTCA AATAAA
SynPro37	AGTTATATTATACAACATAAAAATTGACATGTCA ATTCAC
SynPro42	CGTGCAATTATACCTGGCCGCGAGAGCCTTGTCA ATGGGC
SynPro51	CAACTTATTATACAGTCGCGAATGTCGGATGTCA AGTAGA
Crc site	AAAAACAAGAGGAAAACAA
Crc insensitive site	TCTCTCAGAGGAAAACAA

Summary of Thesis in Spanish by sections

I. Resumen

La Biología Sintética se inspira en los principios de la ingeniería eléctrica e industrial para modificar o diseñar nuevos sistemas biológicos. Al igual que la ingeniería, la Biología Sintética emplea estándares para diseñar y obtener partes genéticas y con ellas generar módulos y sistemas. El uso de herramientas moleculares uniformadas permite comparar resultados en distintos laboratorios, pero requiere la adopción de una nomenclatura común y el desarrollo de plataformas genéticas compartidas. Las bacterias constituyen un sistema óptimo para re-programar o re-implantar módulos genéticos, y *Escherichia coli* es la cepa más utilizada para estas tareas. Las bacterias del suelo, como *Pseudomonas putida* son una alternativa en cuanto que presentan una gran adaptabilidad hacia el ambiente externo. *P. putida* KT2440 es una cepa derivada de un aislado silvestre que degrada tolueno (*P. putida* mt-2) y es el candidato a hospedador con más potencial para aplicaciones industriales y medioambientales de circuitos genéticos hechos con Biología Sintética. La presente Tesis describe un conjunto de herramientas moleculares diseñadas para trabajar con *P. putida* KT2440, con la finalidad de clarificar algunos aspectos sobre las regulación de algunas de sus propiedades y avanzar en su utilización en aplicaciones biotecnológicas. En primer lugar, esta Tesis propone el diseño y la validación de herramientas basadas en plásmidos y transposones estandarizados que permitan efectuar un análisis detallado sobre el estado de activación de promotores específicos en células únicas y en poblaciones. A continuación, se investigan la dinámica de la regulación del nodo transcripcional formado por el complejo XylR/*Pu,* que está implicado en la degradación de compuestos aromáticos en *P. putida*. En particular, se analizan las respuestas del promotor *Pu* en distintas condiciones fisiológicas, como el aumento de la producción de su regulador XylR y la presencia de diferentes inductores químicos. Se analiza además la producción de XylR *in vivo* cuando las células de *P. putida* KT2440 crecen en un régimen de represión catabólica mediado por la proteína Crc. Finalmente, se muestra el potencial del sistema de expresión ChnR/*PchnB* diseñado para obtener condicionalmente biopelículas de *P. putida* KT2440 capaces de degradar compuestos halogenados.

II. Objetivos

El objetivo principal del trabajo es el diseño de herramientas moleculares estándares, para comprender las propiedades regulatorias y bioquímicas de *P. putida* KT2440.

Objetivos específicos

1) Diseñar y validar herramientas genéticas estándares basadas en el funcionamiento de plásmidos y transposons para analizar la expresión génica en bacteria Gram-negativas.
2) Analizar la dinámica de la regulación del nodo transcripcional formado por el complejo XylR/*Pu* XylR/*Pu* que está implicado en la degradación de compuestos aromáticos en *P. putida*.
3) Analizar hla producción de XylR *in vivo* cuando las células de *P. putida* KT2440 crecen en un régimen de represión catabólica mediado por la proteína Crc
4) Mostrar potencial del sistema de expresión ChnR/*PchnB* diseñado para obtener condicionalmente biopelículas de *P. putida* KT2440 capaces de degradar compuestos halogenados.

III. Conclusiones

Los resultados de esta tesis han llevado a las conclusiones siguientes:

1. Las herramientas genéticas diseñadas para bacterias Gram-negativas, basadas en plásmidos y transposones, se han revelado funcionales y útiles para cuantificar la expresión génica en poblaciones y células individuales.
2. El cambio en la concentración del regulador XylR, por ejemplo variación en número de copias, remueve la bimodalida del promotor target *Pu* en células únicas de *P. putida*.
3. Los efectores sub-óptimos de XylR provocan una respuesta bimodal en el promotor *Pu* tanto en población cómo en células únicas de *P. putida.*
4. La proteína Crc no parece manifestar un rol central por inhibir la traducción de XylR en un régimen de represión catabólica en células únicas de *P. putida*
5. *P. putida* KT2440 se puede modificar genéticamente para aplicaciones biotecnológicas, como producir biopelículas capaces de degradar compuestos halogenados.